CEITEC

Central European Institute of Technology

BRNO | CZECH REPUBLIC

Analytical ultracentrifugation

Biomolecular interactions

Jan Komárek

Interacting systems on AUC

SELF-ASSOCIATION (oligomerization)

e.g. $A + A \rightleftarrows A_2$

- two or more identical molecules

HETERO-ASSOCIATION

e.g. $A + B \rightleftarrows AB$

- two or more different proteins
- protein-DNA
- protein-polysaccharide
- ~~protein - small molecule~~

What can be determined?

- stoichiometry (reaction scheme)
- affinity - K_d
- shape of the complex

Models in SEDPHAT

$nA \rightleftarrows A_n$

$A + B + B + C \rightleftarrows \ldots$

$A + A + A \rightleftarrows A_3$

$A \rightleftarrows A_4 \rightleftarrows A_8$

$A + A \rightleftarrows A_2$

$A + B + C \rightleftarrows \ldots$

competing B and C for A

$A + B + B \rightleftarrows ABB$

2 non-symetrical sites

$A \rightleftarrows A_2 \rightleftarrows A_4$

$(A+A) + B + B \rightleftarrows \ldots$

self-associating A with two sites for B

$A + B \rightleftarrows AB$

$A + B + C \rightleftarrows ABC$

$A + B + B + C + C \rightleftarrows \ldots$

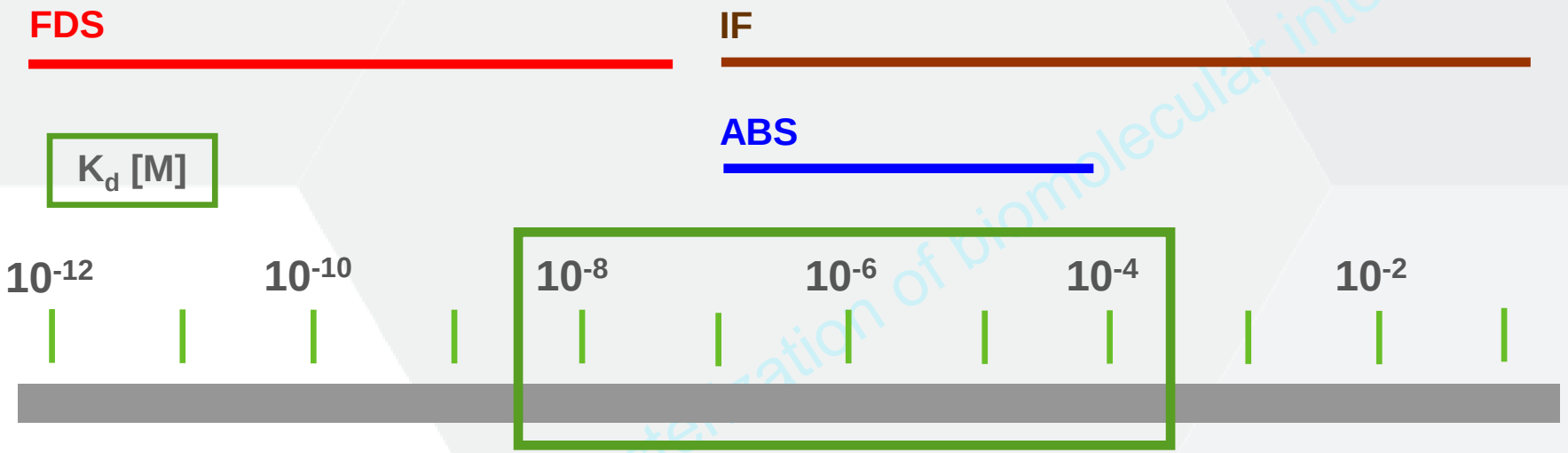
$A + B + B \rightleftarrows ABB$

$(A+A) + (B+B) \rightleftarrows \ldots$

heterodimer of homodimers

$A + B + B + B \rightleftarrows ABBB$

Affinity range of biomolecular interactions studied by AUC



very strong interactions	moderate-strength interactions	weak/very weak interactions
very low concentration, dye-labeled proteins	proteins 0.1 - 5 mg/ml	higher concentrations necessary (≥ 5 mg/ml) – complications due to non-ideality effects

Dissociation constant (K_d)

- equilibrium constant that measures the propensity of a larger object to dissociate reversibly to smaller components
- commonly used to describe **the affinity** between the molecules (lower K_d ~ higher affinity)
- inverse of **association consant** ($1/K_a$)

$$A_xB_y \rightleftharpoons xA + yB \qquad K_d = \frac{[A]^x \cdot [B]^y}{[A_xB_y]}$$

frequently x = y = 1:

$$AB \rightleftharpoons A + B \qquad K_d = \frac{[A] \cdot [B]}{[AB]}$$

Dissociation constant (K_d)

$$AB \rightleftharpoons A + B \qquad K_d = \frac{[A]\cdot[B]}{[AB]}$$

When [A] = K_d, then [B] = [AB] !

mass conservation:

$$[A]_0 = [A] + [AB]$$
$$[B]_0 = [B] + [AB]$$

For a 2-component system, K_d has concentration units (M)

Effect of concentration on species´ populations

Self-association

$A + A \rightleftarrows A2$

$K_d = 1$ uM

total conc. of A	% of A as a monomer	% of A as a dimer
0.1 uM	85	15
0.3 uM	70	30
1 uM	50	50
3 uM	33	67
10 uM	20	80

Hetero-association

$A + B \rightleftarrows AB$

$K_d = 1$ uM

total conc. of A	total conc. of B	% of A in a free form	% of A in a complex	% of B in a free form	% of B in a complex
3 uM	**0.1 uM**	98	2	26	74
3 uM	**0.3 uM**	92	8	26	74
3 uM	**1 uM**	77	23	30	70
3 uM	**3 uM**	43	57	43	57
3 uM	**10 uM**	12	88	74	26

Populations changing in the concentration-dependent manner…

Below K_d, equilibrium shifted towards smaller oligomer (self-assoc.) /free species (hetero-assoc.)

Above K_d, equilibrium shifted towards higher oligomer (self-assoc.) /complex (hetero-assoc.)

Determination of K_d

Self-association

$A + A \rightleftarrows A2$

$K_d = 1$ uM

total conc. of A	% of A as a monomer	% of A as a dimer
0.1 uM	85	15
0.3 uM	70	30
1 uM	50	50
3 uM	33	67
10 uM	20	80

10x

10x

→ **Dilution series**

Hetero-association

$A + B \rightleftarrows AB$

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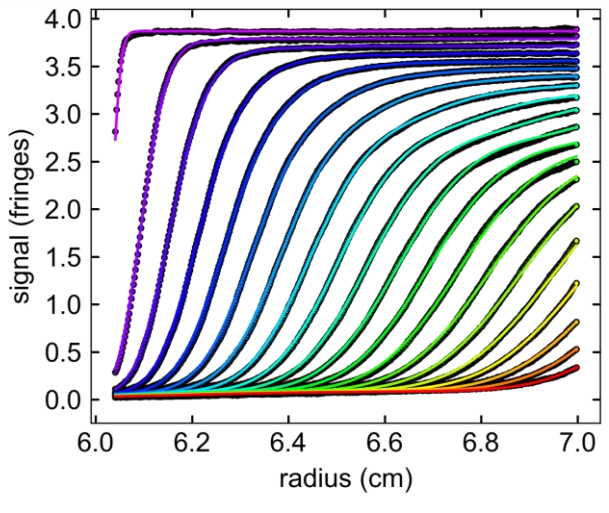
→ **Titration series (A with B and/or B with A)**

Dilution series of a purified complex

Using a broad concentration range (one order above and below K_d) for accurate K_d determination!

Question: Any idea on K_d of BSA?

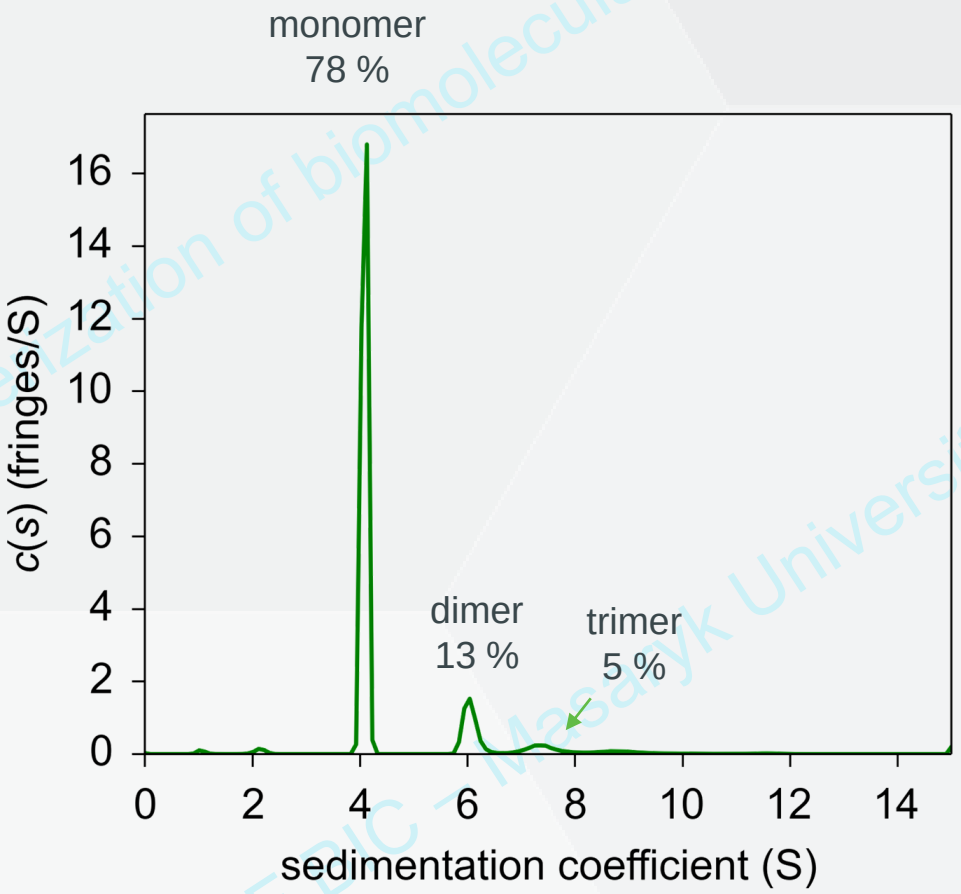
raw SV data:



48,000 rpm, 20 °C
IF detection

BSA, 1 mg/ml
PBS buffer, pH 7.5

c(s) distribution of 16 uM BSA:



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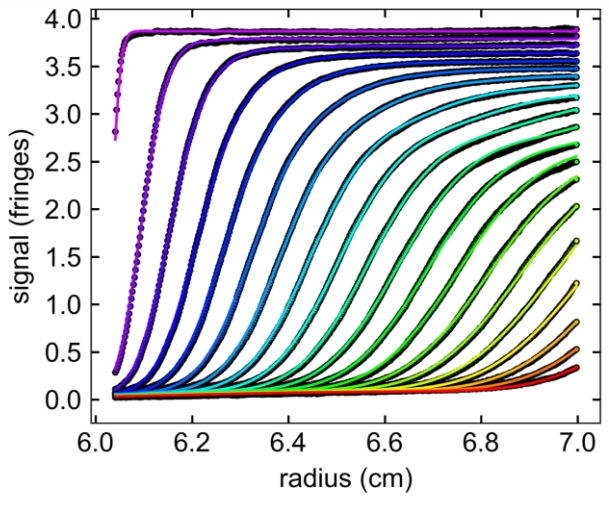

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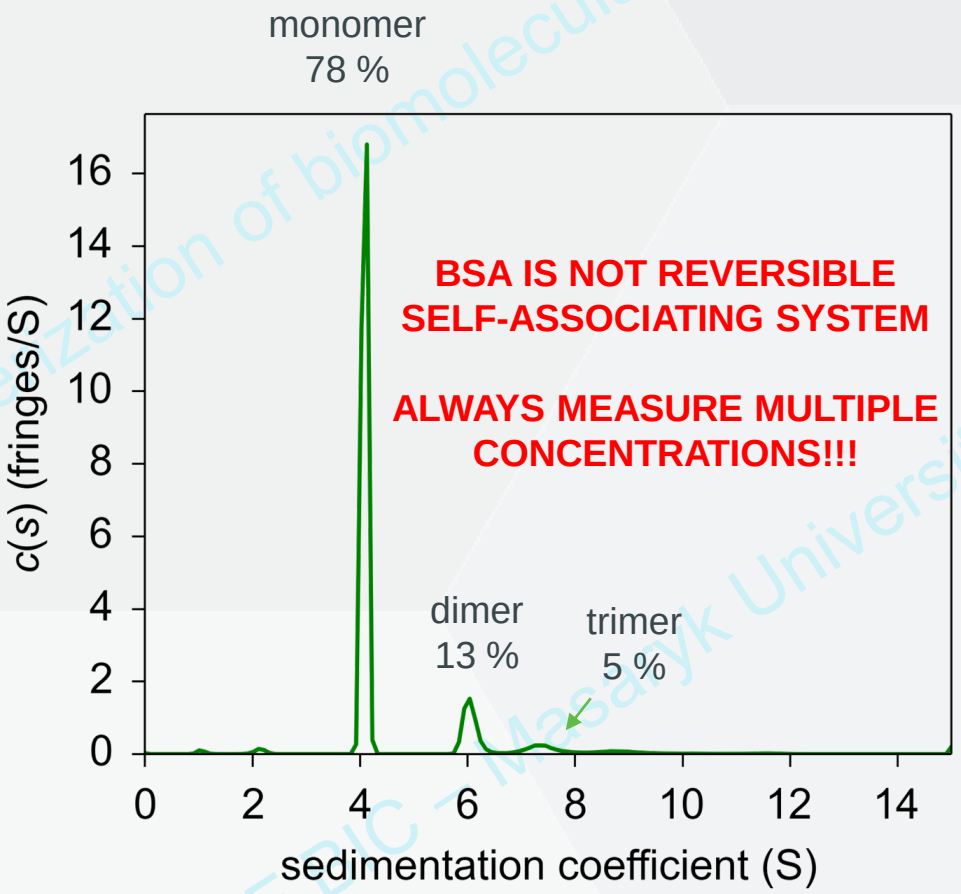


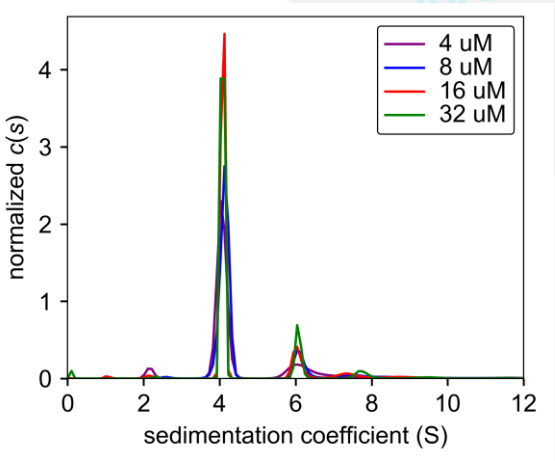
General workflow

STEP 1: Performing SV experiment at different loading concentration/ molar ratios + comparing c(s) distributions

Is there a reversible interaction?

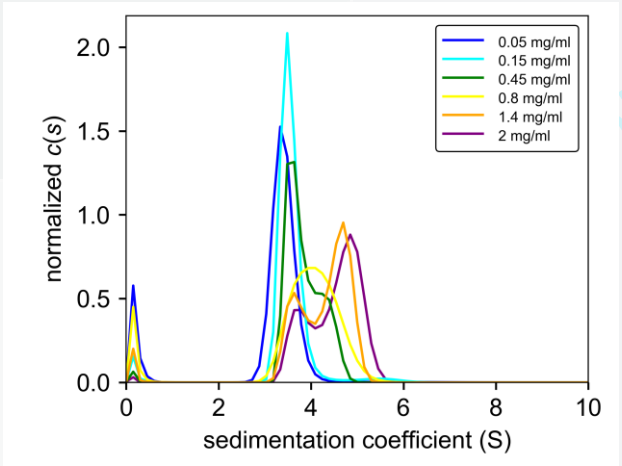
- emerging of new peaks in c(s) distribution?
- shifts in peak position with protein concentration?
- changes in peak areas with protein concentration?

BSA



x

His kinase



General workflow

STEP 1: **Performing SV experiment at different loading concentration/ molar ratios + comparing c(s) distributions**

Fast or slow interaction?

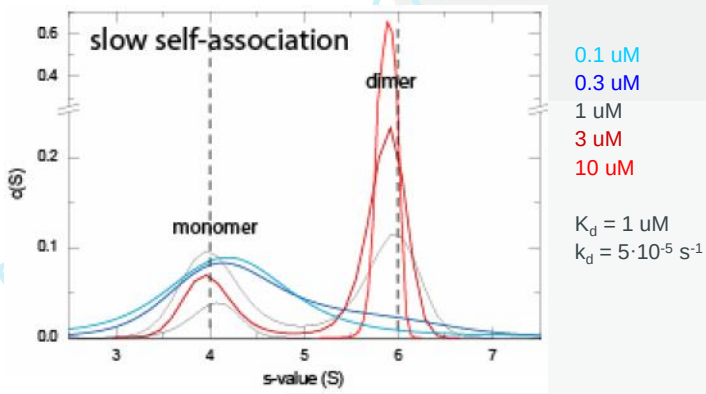
Self-association (monomer-dimer)

SLOW INTERACTIONS

($k_d < 10^{-3}$-10^{-4} s^{-1})

$2A \rightleftharpoons A_2$

Sedimenting species stable, peak positions constant, relative peak areas change with increasing concentration

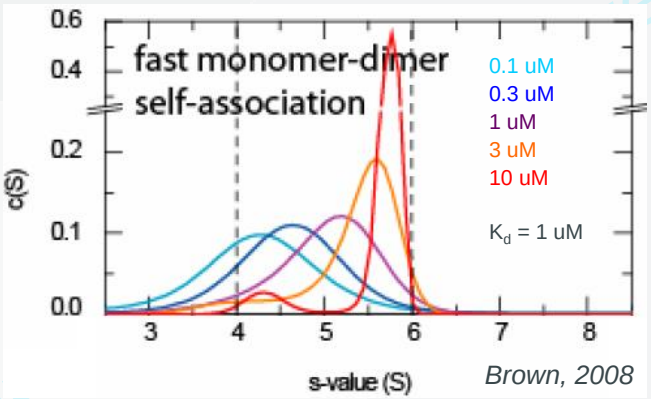


FAST INTERACTIONS

($k_d > 10^{-3}$ s^{-1})

$2A \rightleftharpoons A_2$

Rapid interconversion between complex and free species, peak position change with increasing concentration



Brown, 2008

General workflow

STEP 1: Performing SV experiment at different loading concentration/ molar ratios + comparing c(s) distributions

Fast kinetics system:

Sedimentation of A, B and complex AB

A =

B =

AB =

Schuck, 2010

General workflow

STEP 1: **Performing SV experiment at different loading concentration/ molar ratios + comparing c(s) distributions**

Fast or slow interaction?

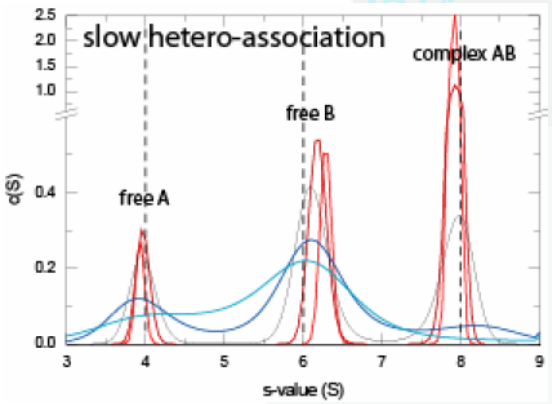
Hetero-association (A + B → AB)

SLOW INTERACTIONS

($k_d < 10^{-3}$-10^{-4} s^{-1})

A + B ⇌ AB

Sedimenting species stable, peak positions constant, relative peak areas change with increasing concentration



0.1 uM
0.3 uM
1 uM
3 uM
10 uM

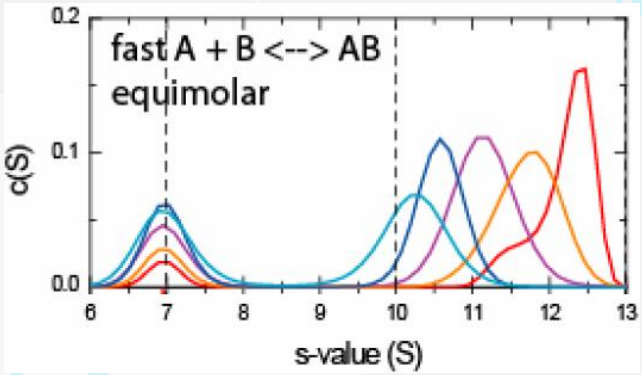
K_d = 1 uM
$k_d = 5 \cdot 10^{-5}$ s^{-1}

FAST INTERACTIONS

($k_d > 10^{-3}$ s^{-1})

A + B ⇌ AB

Rapid interconversion between complex and free species, peak position change with increasing concentration



0.1 uM
0.3 uM
1 uM
3 uM
10 uM

K_d = 1 uM

Brown, 2008

General workflow

STEP 2: Analyzing the interaction, determination of K_d

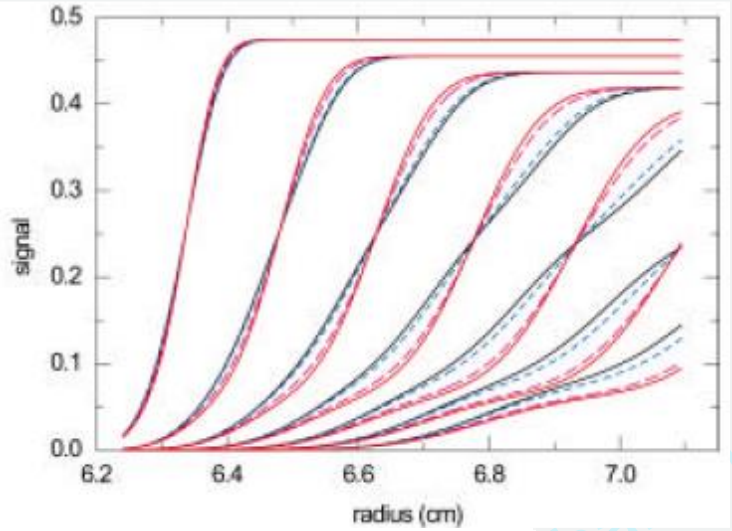
SEDIMENTATION VELOCITY

- direct fitting of sedimentation boundaries
- analysis with binding isotherms
- multi-signal SV (MSSV technique) – hetero-associations only

SEDIMENTATION EQUILIBRIUM

Direct fitting approach

Lamm equation for interacting system (1:1 hetero-association):



Balbo, 2006

$$\frac{\partial c_i}{\partial t} = \frac{1}{r}\frac{\partial}{\partial r}\left[rD_i\frac{\partial c_i}{\partial r} - s_i\omega^2 r^2 c_i\right] + \sigma_i q, \quad i = 1, 2, 3$$

$$q = \frac{k_{\text{off}}}{K_{\text{d}}} c_1 c_2 - k_{\text{off}} c_3 \quad \text{and} \quad \sigma_1 = \sigma_2 = -\sigma_3 = -1$$

q – chemical flux, *1* and *2* are free species, *3* is a complex

- necessary to globally fit the data obtained at different concentrations/molar ratios
- difficult to apply in practice
- high requirements on sample purity

Analysis with binding isotherms

ISOTHERM – *„dependence of a physical property of a mixture of interacting components on the loading concentrations (keeping all other parameters constant, including temperature)“*

Some macroscopis observations of a mixture are dependent on the relative concentration of free and complex species, and a set of measurements in a concentration series generate an isotherm that can be analyzed to determine reaction scheme and K_d

s_w – weight-averaged s-value of the whole system as a function of loading concentration of sample (integration of distribution over all species participating in interaction)

$s_{w,fast}$ – weight-averaged s-value of the reaction boundary as a function of loading concentration (fast kinetics only)

pop – concentration-dependent shift of peak areas (populations)

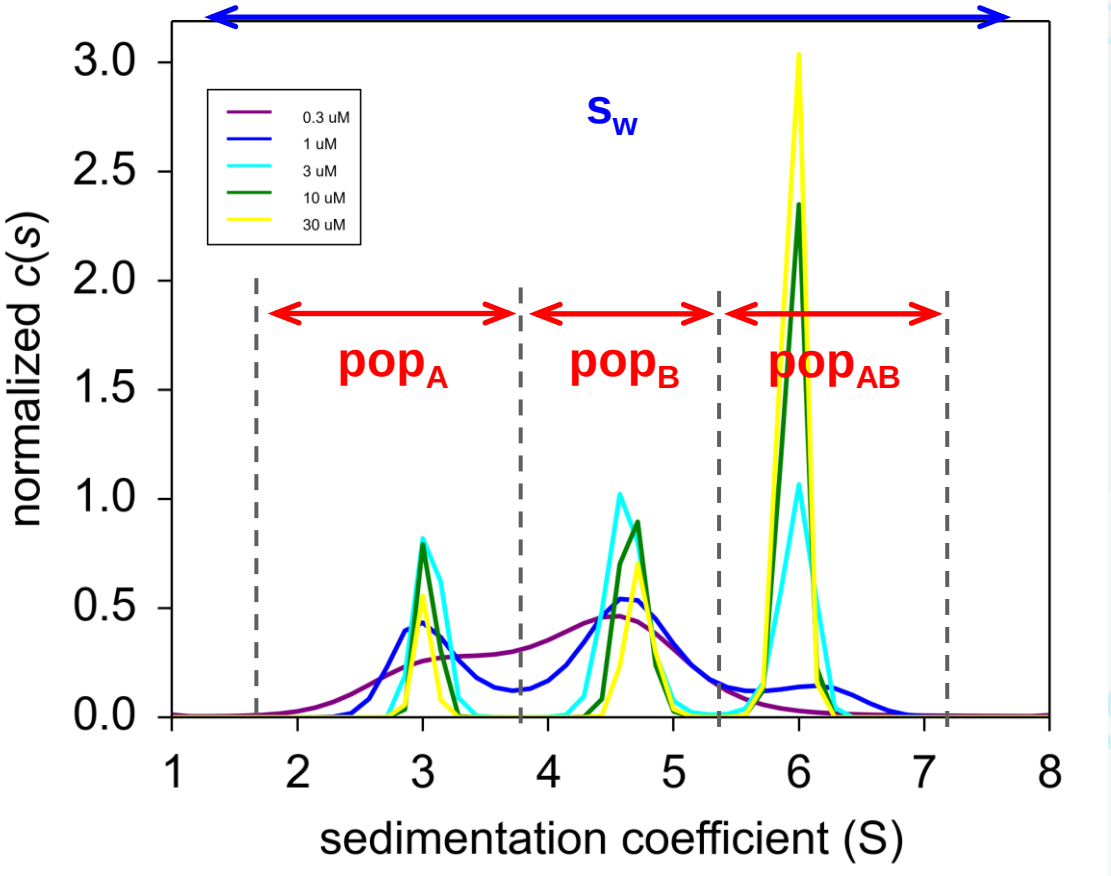
Analysis with binding isotherms:

- most frequently used approach, more tolerant to impurities
- information extracted from the c(s) distributions

Analysis with binding isotherms

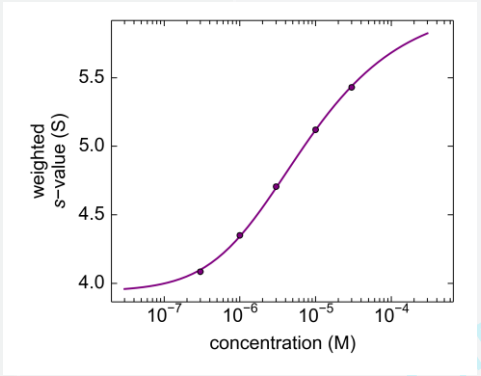
Example of analysis – A + B ⇄ AB with slow kinetics

Different concentrations of proteins – dilution serie, A and B in equimolar concentrations

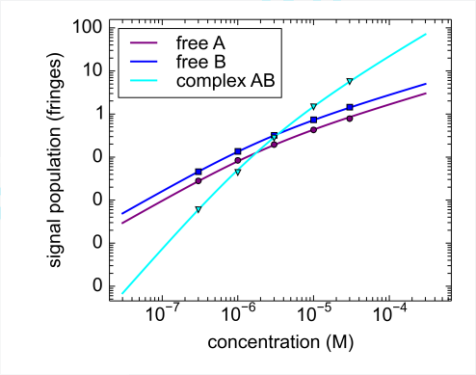


fitting of data to A+B ⇄ AB model for binding isotherms

s_w isotherm



pop isotherms

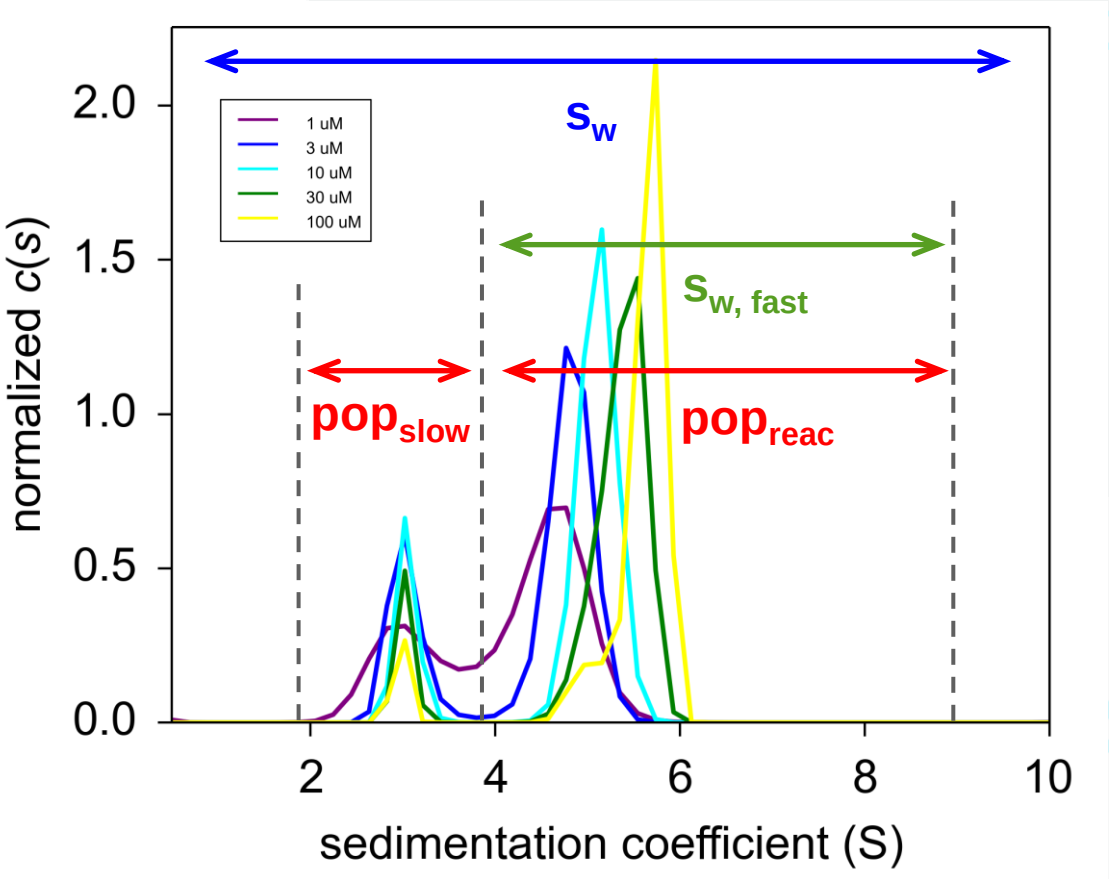


K_d = 3.4 µM

Analysis with binding isotherms

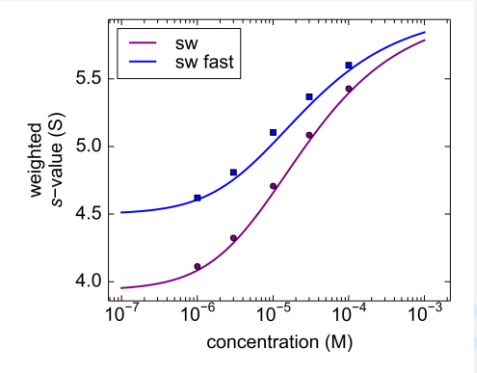
Example of analysis – A + B ⇄ AB with fast kinetics

Different concentrations of proteins – dilution serie, A and B in equimolar concentrations

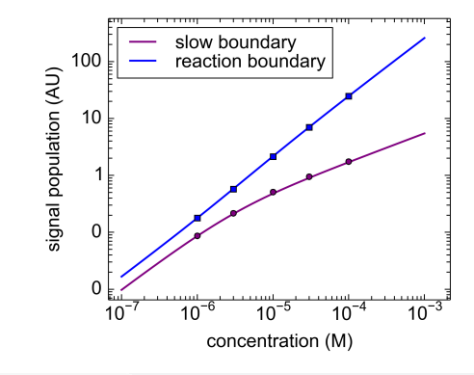


fitting of data to A+B ⇄ AB model for binding isotherms

s_w and $s_{w,fast}$ isotherm



pop isotherms



K_d = 12.2 µM

Real example – K_d determination

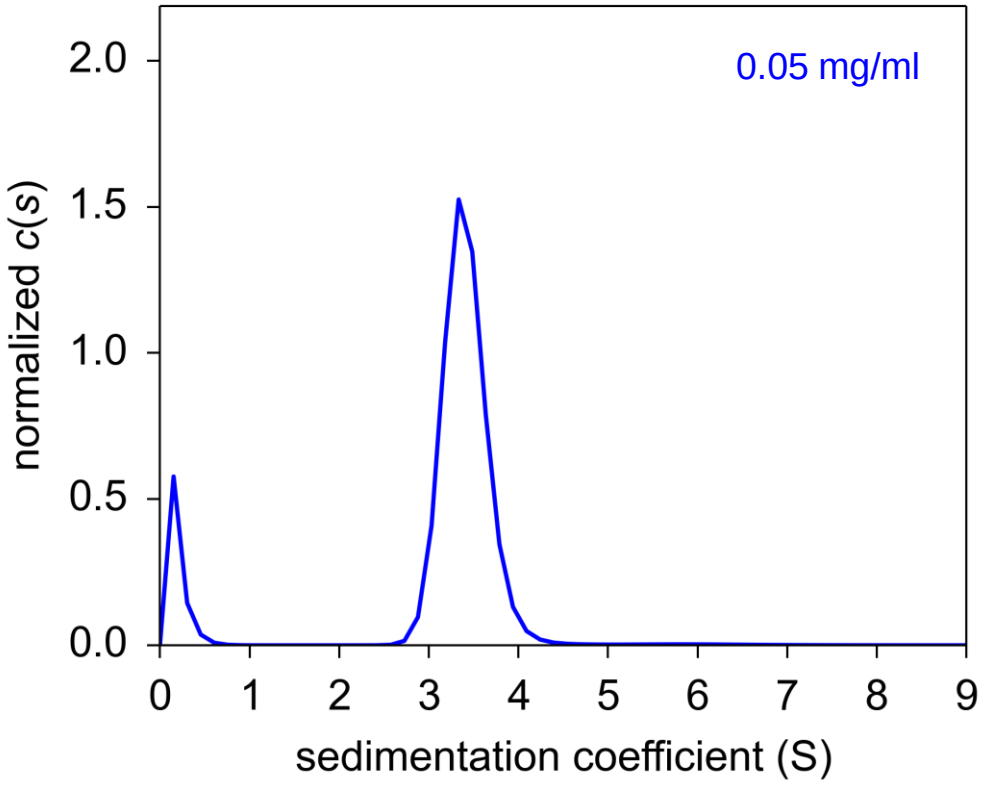
His kinase

Example of analysis of an interacting system

Oligomerization of histidine kinase:

Sedimentation velocity AUC:

20º C, 48000 rpm, ABS detection (230 nm or 280 nm)
20 mM Tris/HCl, 100 mM NaCl, pH 7.4



SV experiment performed at different loading concentrations of protein.

The distributions do not overlay, there is a shift to higher s with increasing concentration.

= sign of reversible interactions

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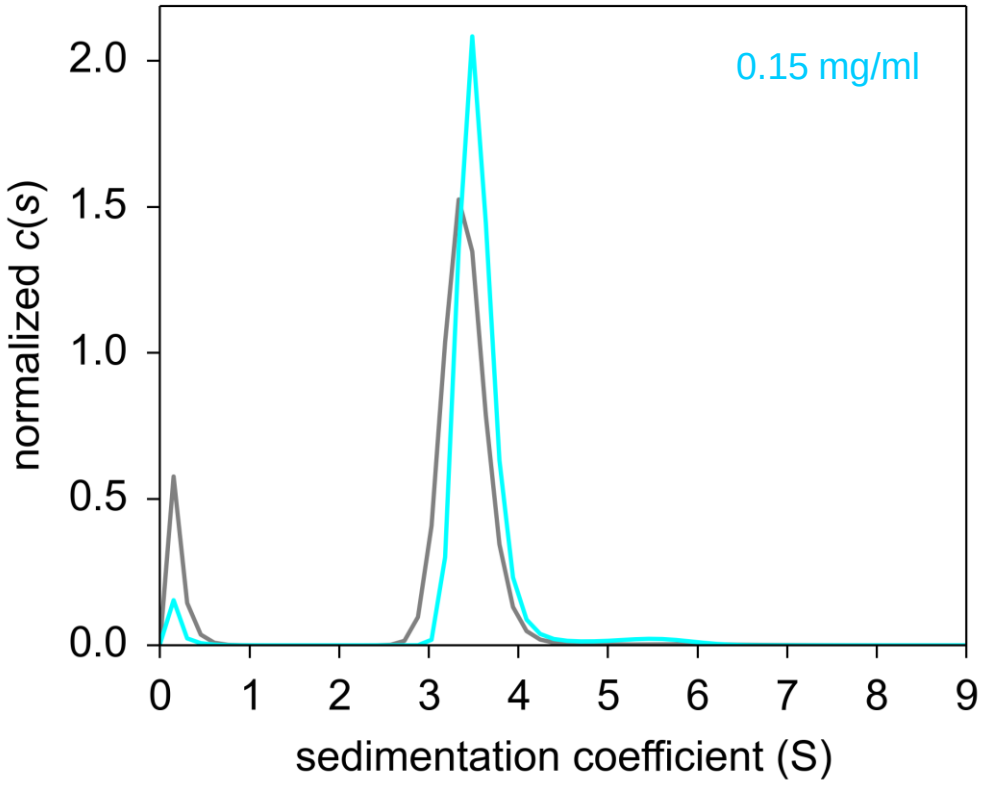

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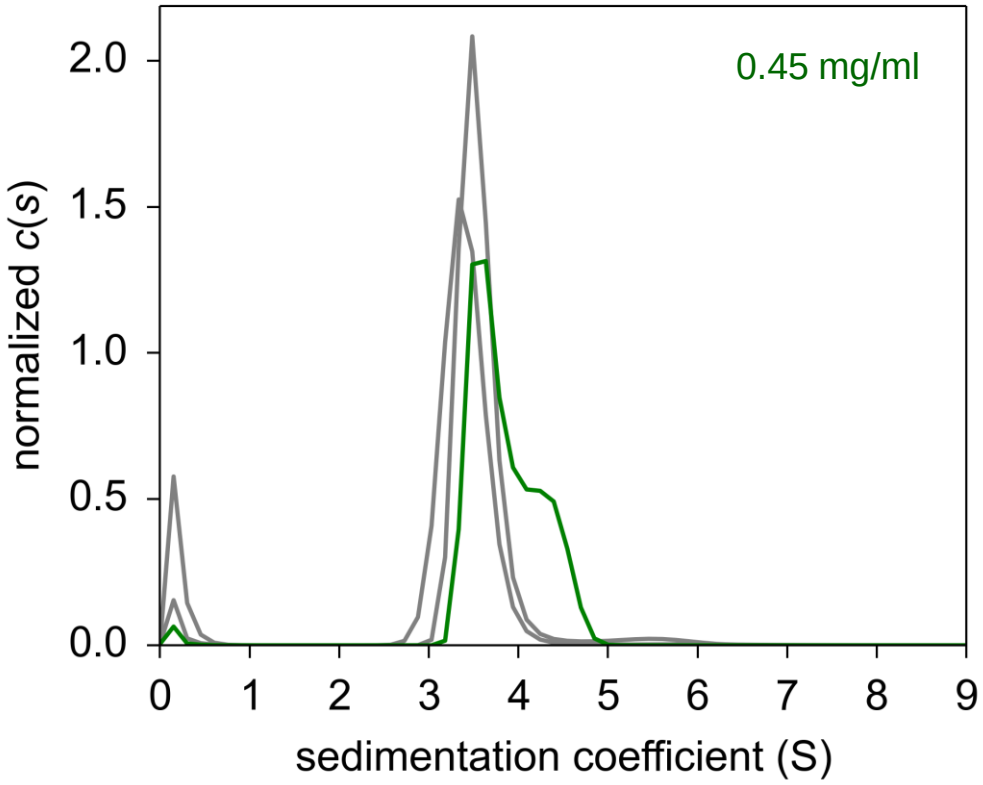

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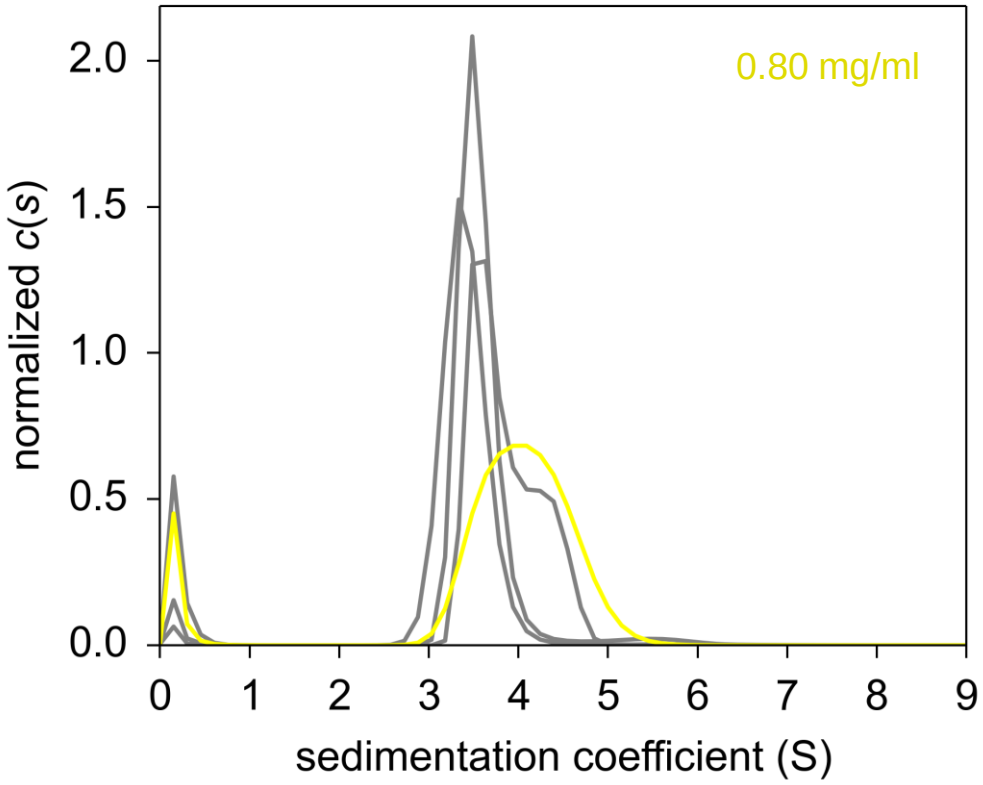

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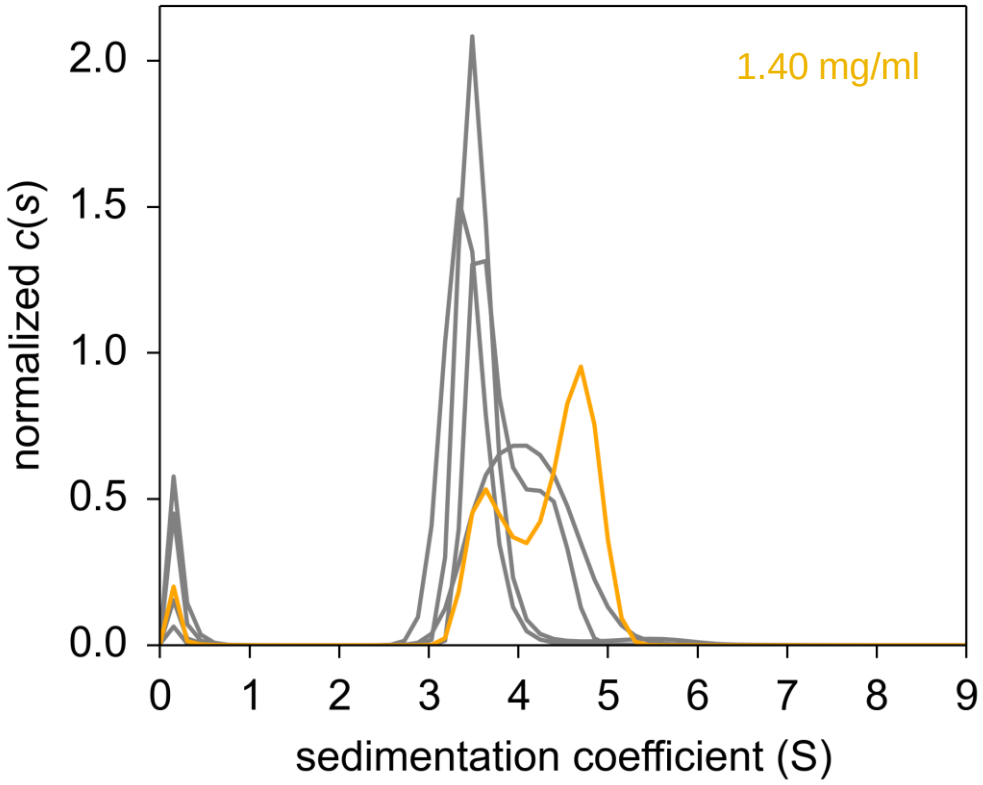

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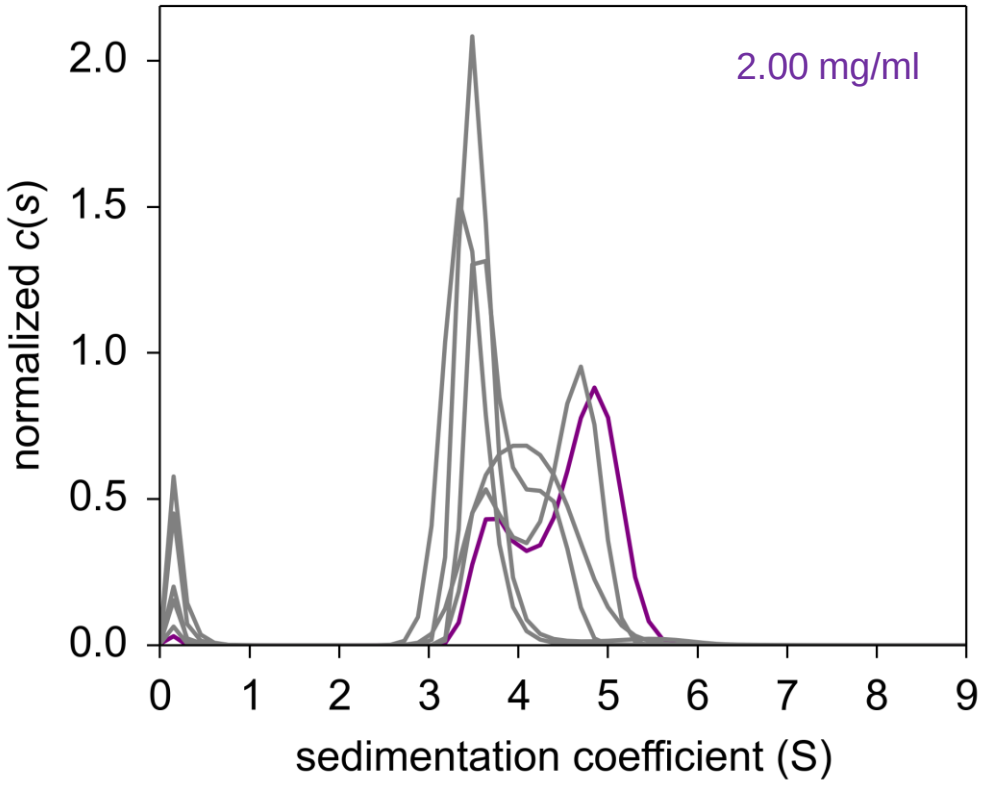

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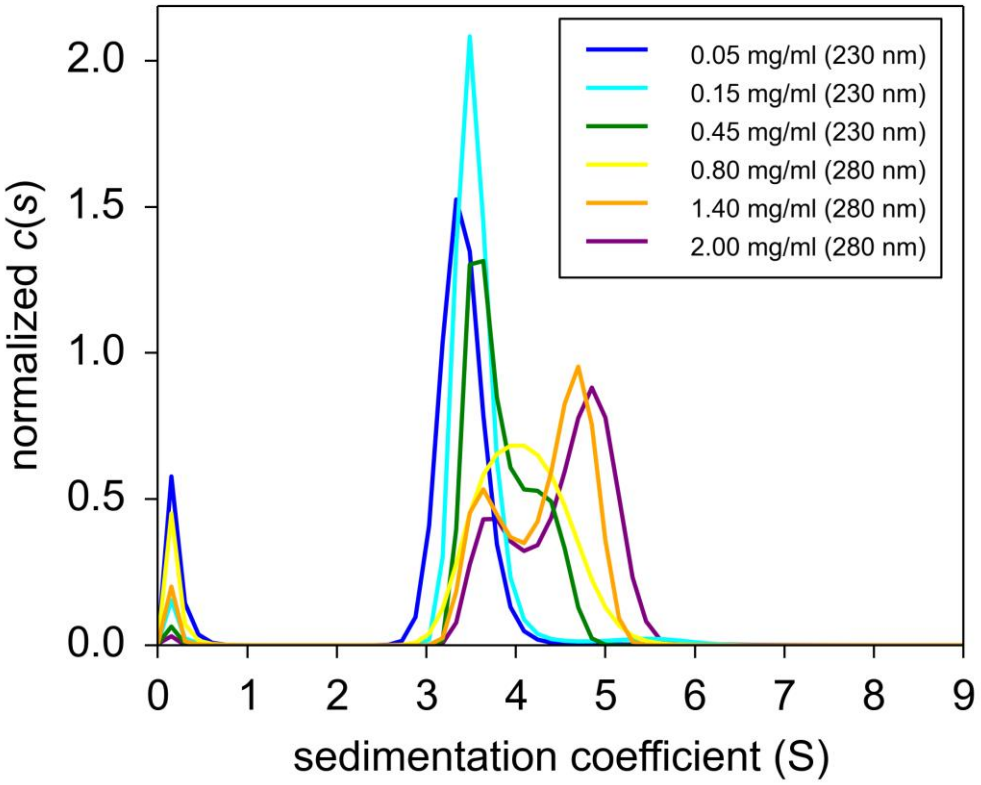

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REVERSIBLE DIMER-TETRAMER EQUILIBRIUM

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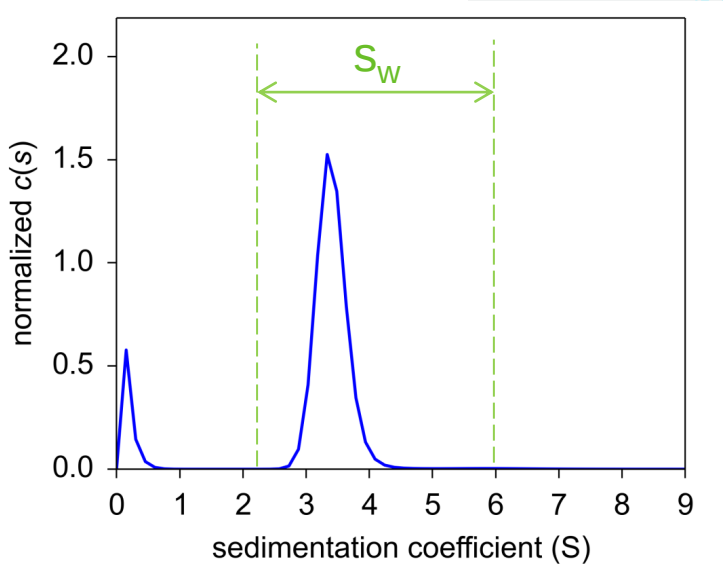
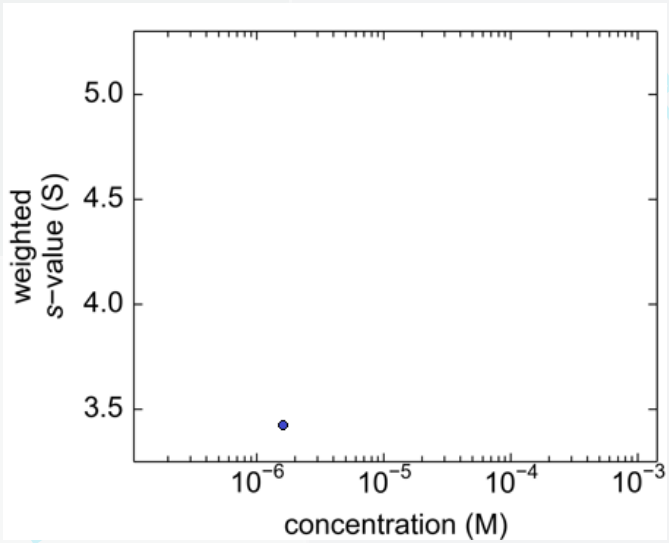
s_w isotherm analysis:



0.05 mg/ml:
s_w = 3.425 S



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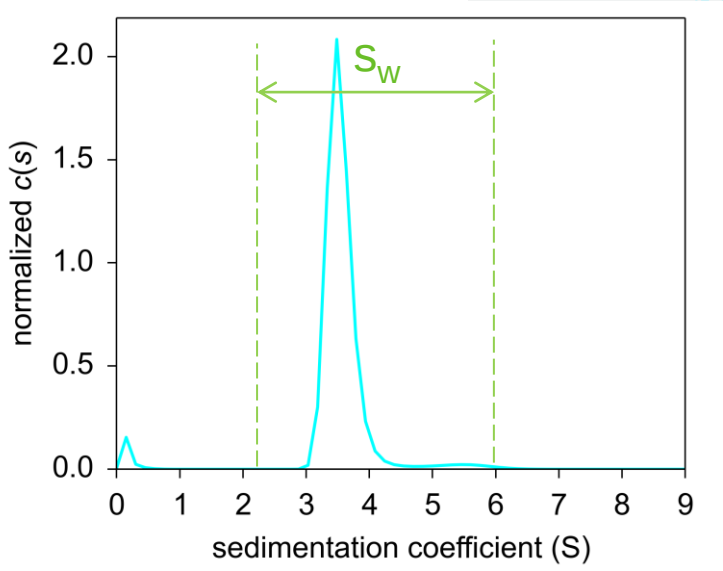
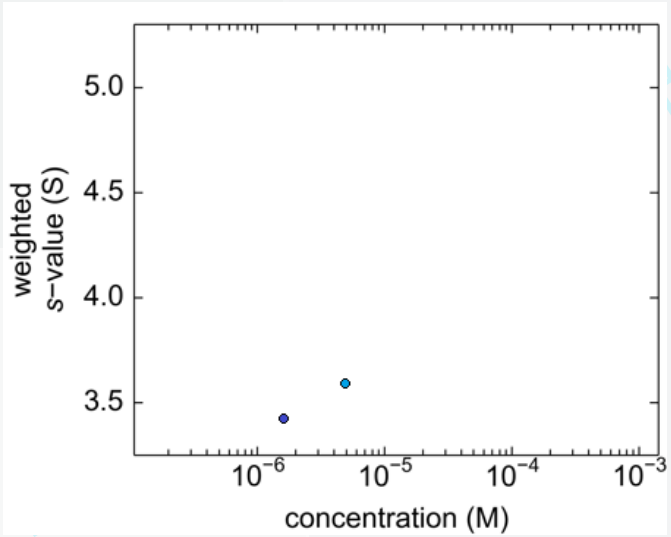


0.15 mg/ml:
s_w = 3.589 S



Example of analysis of an interacting system

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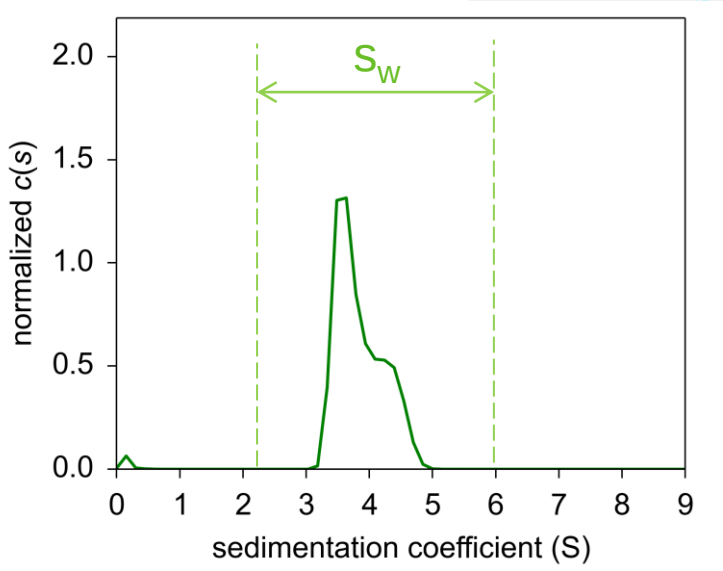
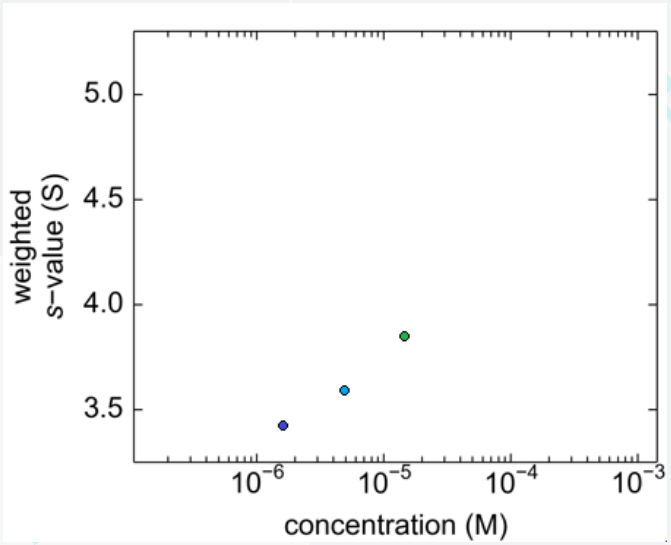
20 mM Tris/HCl, 100 mM NaCl, pH 7.4

s_w isotherm analysis:



0.45 mg/ml:
s_w = 3.850 S



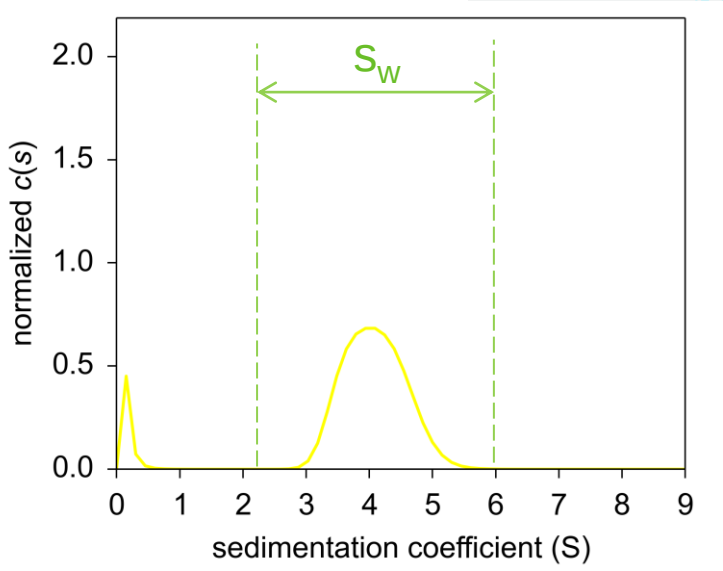
Example of analysis of an interacting system

Oligomerization of histidine kinase:

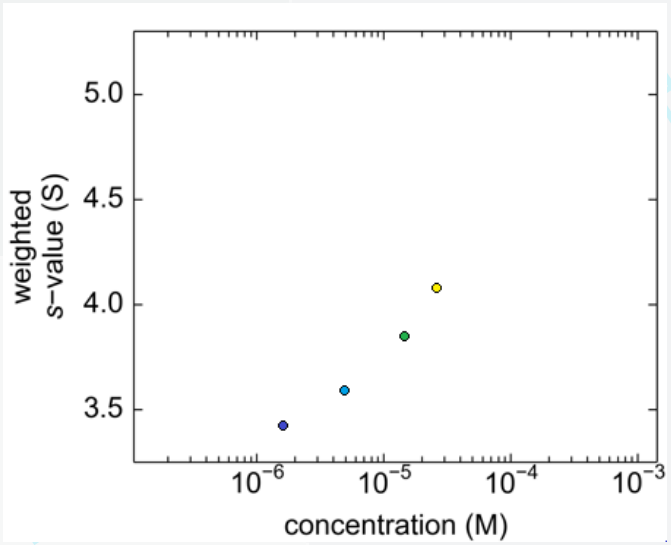
Sedimentation velocity AUC:

20º C, 48000 rpm, ABS detection (230 nm or 280 nm)
20 mM Tris/HCl, 100 mM NaCl, pH 7.4

s_w isotherm analysis:



0.80 mg/ml:
s_w = 4.079 S



Example of analysis of an interacting system

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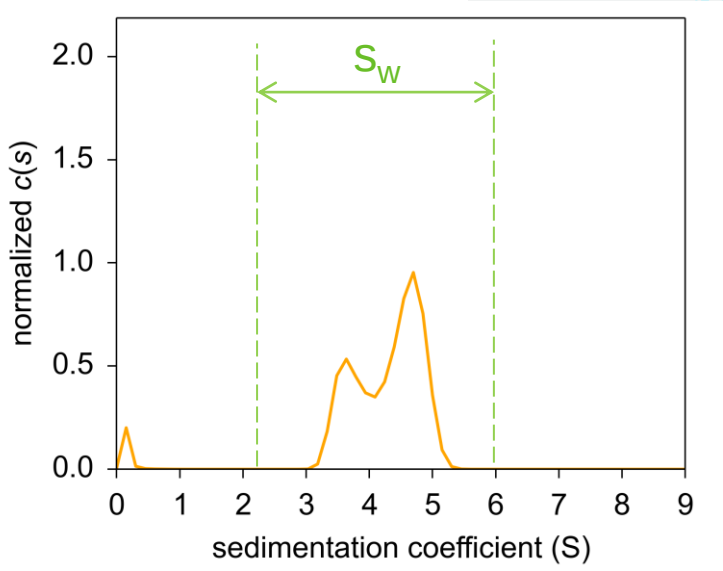
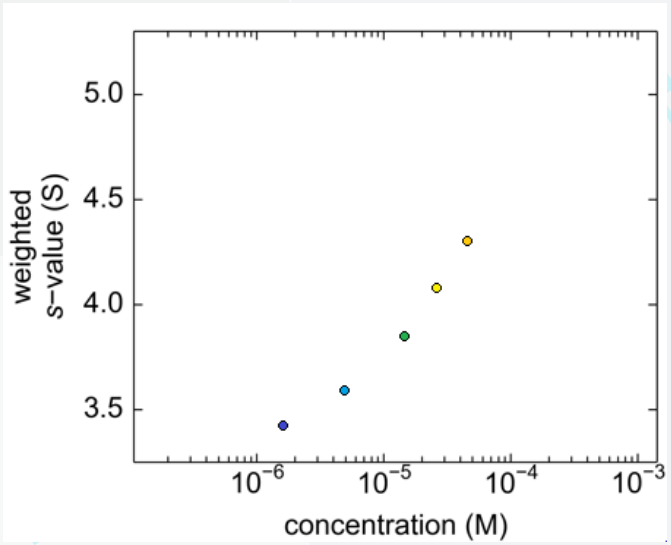


1.40 mg/ml:
s_w = 4.307 S



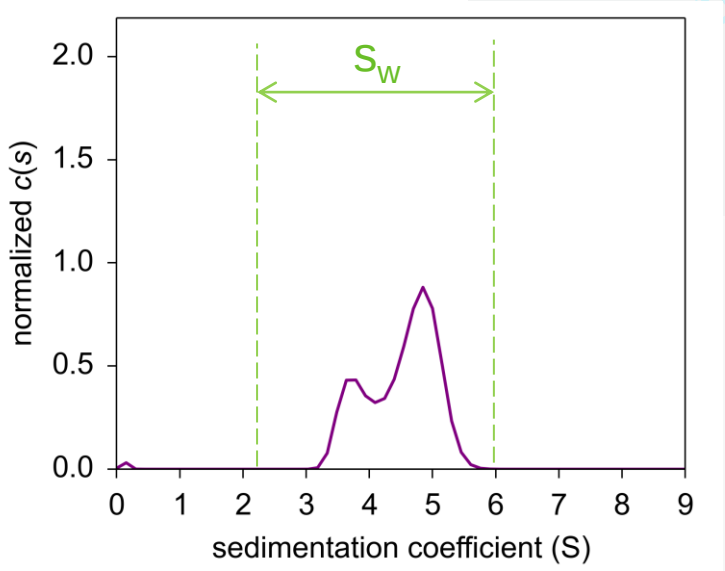
Example of analysis of an interacting system

Oligomerization of histidine kinase:

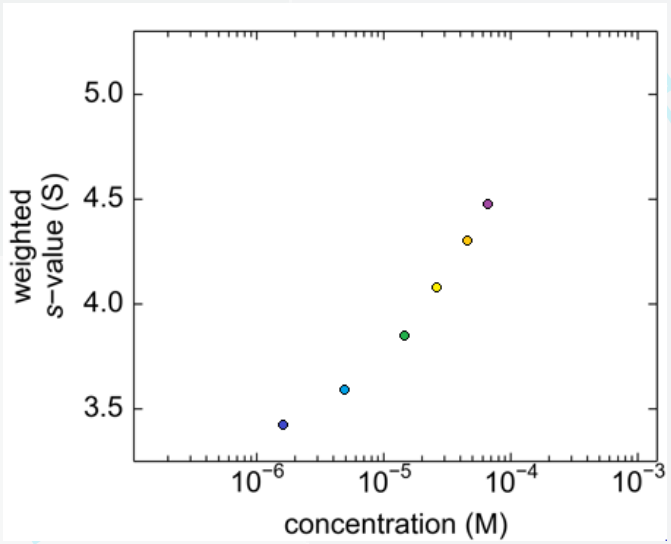
Sedimentation velocity AUC:

20º C, 48000 rpm, ABS detection (230 nm or 280 nm)
20 mM Tris/HCl, 100 mM NaCl, pH 7.4

s_w isotherm analysis:



2.00 mg/ml:
s_w = 4.476 S



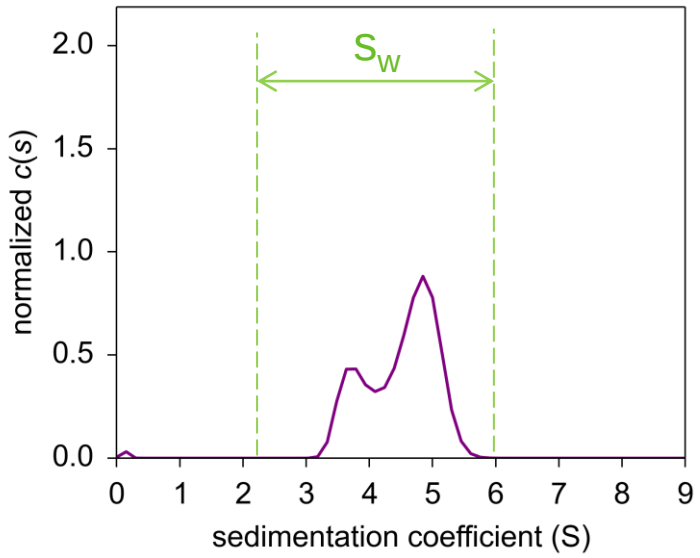
Example of analysis of an interacting system

Oligomerization of histidine kinase:

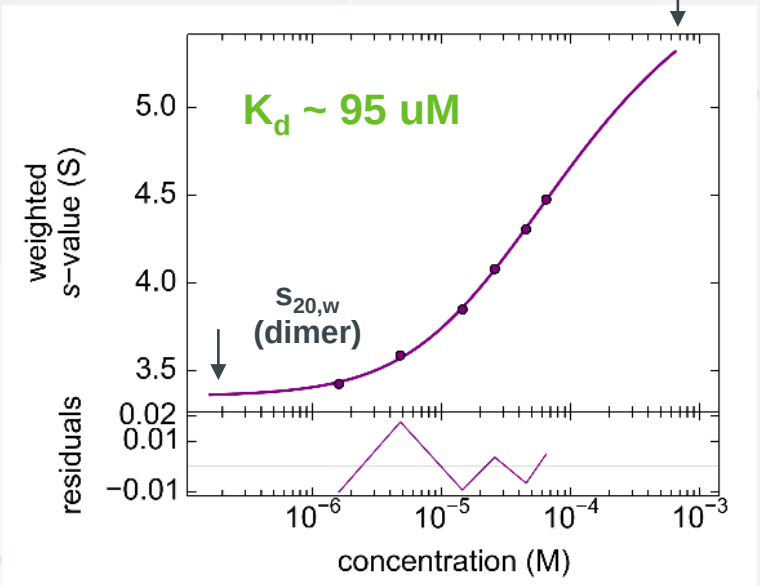
Sedimentation velocity AUC:

20º C, 48000 rpm, ABS detection (230 nm or 280 nm)
20 mM Tris/HCl, 100 mM NaCl, pH 7.4

s_w isotherm analysis:

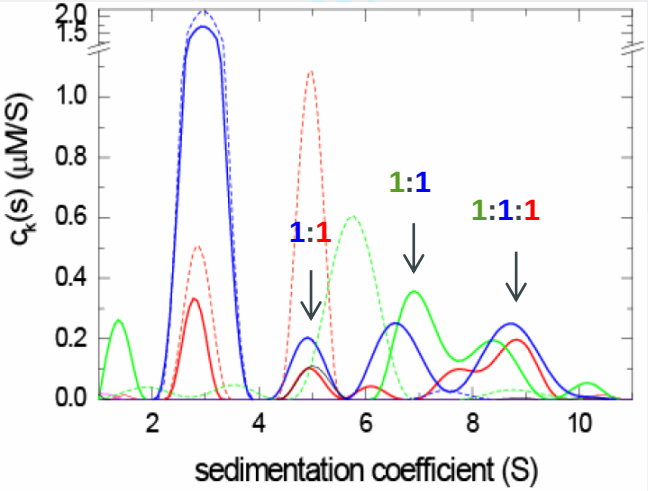


2.00 mg/ml:
s_w = 4.476 S



Multi-signal sedimentation velocity (MSSV)

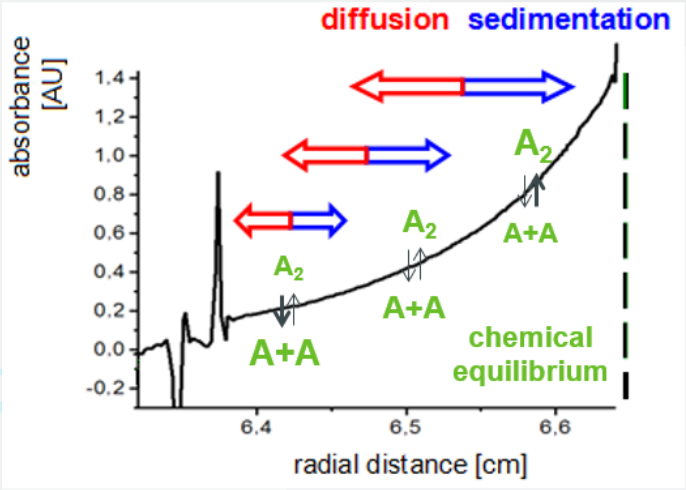
- determination of complex stoichiometry in heterogeneous interactions of species with significantly different spectral properties ($\varepsilon_{280}/\varepsilon_{250}$, $\varepsilon_{280}/\varepsilon_{IF}$)
- acquisition of multiple signals (wavelengths) necessary
- useful even for impure samples, multi-step associations, or in cases the correct reaction model is unknown



MSSV analysis of triple protein mixture of viral glycoprotein, its cognate receptor and antigen-recognition receptor fragment, $c_k(s)$ of mixture shown as solid lines, $c_k(s)$ of each protein alone dotted (*Brown, 2008*)

Sedimentation equilibrium

- sedimentation + diffusion + physical association - all in equilibrium
- high sample purity crucial
- global fitting of data obtained for different loading concentration/molar ratios needed for accurate K_d determination



Collected SE data are fitted with the appropriate model to obtain K_d

$2A \rightleftarrows A_2$

$$c_{tot}(r) = \underbrace{c_1(r_0)\exp\left[M_1^*\frac{\omega^2(r^2-r_0^2)}{2RT}\right]}_{A} + \underbrace{K_{12}c_1^2(r_0)\exp\left[2M_1^*\frac{\omega^2(r^2-r_0^2)}{2RT}\right]}_{A_2}$$

$$c_2 = K_{12}c_1^2$$

$$M^* = M(1-\bar{v}\rho)$$

M^* - buoyant molar mass

$A + B \rightleftarrows AB$

$$c_{tot}(r) = \overbrace{c_A(r_0)\exp\left[M_A^*\frac{\omega^2(r^2-r_0^2)}{2RT}\right]}^{A} + \overbrace{c_B(r_0)\exp\left[M_B^*\frac{\omega^2(r^2-r_0^2)}{2RT}\right]}^{B} + \underbrace{K_{AB}c_A(r_0)c_B(r_0)\exp\left[(M_A^*+M_B^*)\frac{\omega^2(r^2-r_0^2)}{2RT}\right]}_{AB}$$

$$c_{AB} = K_{AB}c_Ac_B$$

$c(r)$ – concentration of molecule at radial distance r, $c(r_0)$ – concentration of molecule at reference position r_0, K_{12} and K_{AB} – association equilibrium constant [M^{-1}]

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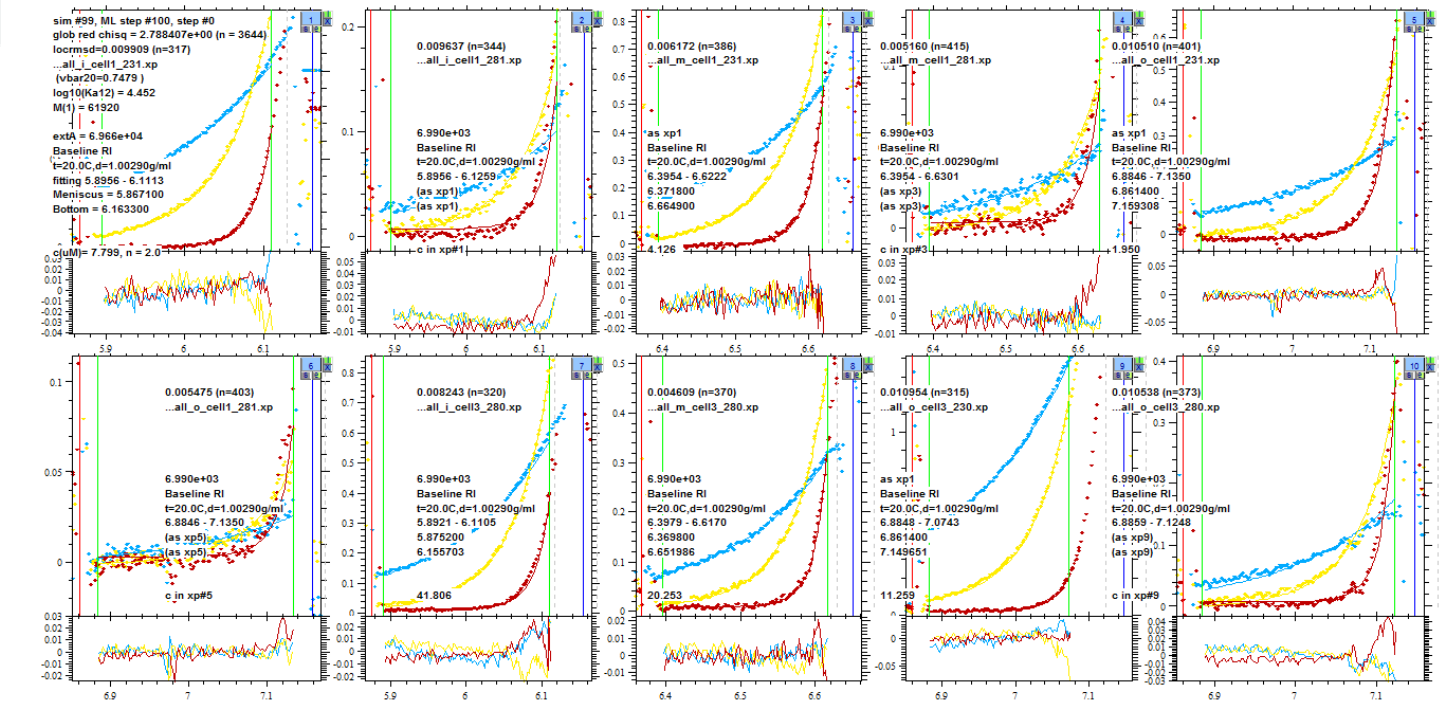
Sedimentation equilibrium

Example of stoichiometry + K_d determination

20º C, 10500 rpm, 17500 rpm, 30000 rpm, ABS detection (230 nm and 280 nm)
20 mM Tris/HCl, 100 mM NaCl, pH 7.4



Concentrations used:

0.06 mg/ml
0.12 mg/ml
0.24 mg/ml
0.35 mg/ml
0.71 mg/ml
1.30 mg/ml

dimer-tetramer model fitting the data well

K_d = 35.3 uM

global analysis of SE data collected at three rotor speeds, 6 different protein concentrations (detection at 280 and 230 nm)

Thank you for your attention

Contact:

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jan.komarek@ceitec.cz

CIISB Czech Infrastructure for Integrative Structural Biology

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