AUC

Olwyn Byron
University of Glasgow, Scotland UK
<table>
<thead>
<tr>
<th>Dates</th>
<th>Positions</th>
<th>Places</th>
</tr>
</thead>
<tbody>
<tr>
<td>84-87</td>
<td>Physics BSc</td>
<td>U. Durham</td>
</tr>
<tr>
<td>87-88</td>
<td>Medical Physics MSc</td>
<td>U. Aberdeen</td>
</tr>
<tr>
<td>88-92</td>
<td>Physical Biochemistry PhD</td>
<td>U. Nottingham &amp; Celltech plc</td>
</tr>
<tr>
<td>92-97</td>
<td>Lecturer</td>
<td>U. Leicester</td>
</tr>
<tr>
<td>97-02</td>
<td>Lecturer</td>
<td></td>
</tr>
<tr>
<td>02-03</td>
<td>Senior Lecturer</td>
<td></td>
</tr>
<tr>
<td>03-04</td>
<td>Maternity leave (14 months)</td>
<td>U. Glasgow</td>
</tr>
<tr>
<td>04-08</td>
<td>Senior Lecturer 0.6 FTE</td>
<td></td>
</tr>
<tr>
<td>08-16</td>
<td>Senior Lecturer</td>
<td></td>
</tr>
<tr>
<td>16-</td>
<td>Professor</td>
<td></td>
</tr>
</tbody>
</table>
The analytical ultracentrifuge (AUC) was invented by Theodor (The) Svedberg.

Nobel Prize in Chemistry 1926 awarded to The Svedberg "for his work on disperse systems"
Svedberg was an interesting man...

- Married 4 times
- 12 children!
- Liked to paint
  - “Atomics”
1960's-80's: AUC = core biochemical /biophysical technology

- Advice from the Beckman Model E AUC 1964 manual:
- “The Model E, like a woman, performs best when you care. But you needn’t pamper it - just give it the understanding it deserves.”

http://ultrascan.aucsolutions.com/
AUC is hugely complimentary to SAS

You're lovely!!!
AUC is hugely complementary to SAS

<table>
<thead>
<tr>
<th>SAS</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particularly sensitive to large species</td>
<td>“Sees” nearly everything</td>
</tr>
<tr>
<td>Fractionates by interaction with matrix</td>
<td>Separates independently of matrix</td>
</tr>
<tr>
<td>Measures $R_g$, $M$</td>
<td>Measures $s$, $D$, $M$</td>
</tr>
</tbody>
</table>

AUC provides constraints for SAS
Outline

- AUC background
- Experimental considerations
- Sedimentation equilibrium
- Sedimentation velocity
- Hydrodynamic modelling
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AUC tutorials & references

- Tutorial paper

- AUC user guide from Demeler lab

- Book
Alexander Bepperling
• Aggregation analysis and beyond analytical ultracentrifugation in the biopharmaceutical industry
  • https://www.youtube.com/watch?v=liERbl-Xz4c

Borries Demeler
• Advances in sedimentation analysis
  • https://www.youtube.com/watch?v=zuAwWOJZtkM

Chad Brautigam
• Exploring the stoichiometry of macromolecular complexes using multi signal sedimentation velocity analytical ultracentrifugation
  • https://www.youtube.com/watch?v=ea6tvKF8zkA

John Burgner
• Quantitative determination of reaction stoichiometry, interaction energies, and something else
  • https://www.youtube.com/watch?v=ivRodzqWjS8

Andrew Herr
• Analytical ultracentrifugation as a complementary technique for structural analysis of proteins and macromolecular complexes
  • https://www.youtube.com/watch?v=Kw72fyaiQsw
Almost all AUC data analysis software is freely available – here are the most widely used:

- The RASMB website
  - “Reversible Associations in Structural and Molecular Biology”
  - [http://www.rasmb.org/](http://www.rasmb.org/)
  - Access to freely available software
  - Subscription to AUC-related discussion group

- Schuck lab (SEDFIT, SEDPHAT)
  - [http://www.analyticalultracentrifugation.com/default.htm](http://www.analyticalultracentrifugation.com/default.htm)

- Demeler lab (UltraScan III)
  - including US-SOMO
Questions that can be answered by AUC

- Is sample heterogeneous?
  - in molecular weight, shape, or both?
  - does it depend on pH, salt, buffer…??
- Is sample ready for MX, NMR…?
- Does sample…
  - …self-associate?
  - …bind ligand?
    - What is n, Kd?
    - …aggregate?
- What is MW of sample / mix of samples?
- Is association state / conformation affected by tagging?
More questions that can be answered by AUC

- What is sedimentation & diffusion coefficient?
  - Globular / unfolded/disordered?
  - Is conformation dependent on salt, pH, ligand concentration, D...?
- Do mutations affect Kd, conformation, n...?
- Is sample affected by crowding?
Advantages of AUC

- In solution
- Non-destructive
- Self-cleaning
- Absolute
- Complementary
- Can analyse (nearly) anything
  - Proteins
  - Nucleic acids
  - Carbohydrates
  - Polymers
  - Colloids
  - Complexes
  - Nanoparticles
AUC: a high speed preparative UC with optics

Beckman Coulter Optima AUC
Inside the Beckman Coulter XL-I rotor chamber

http://ultrascan.aucsolutions.com/
Inside the Beckman Coulter XL-I

- Vacuum chamber
- Rotor
- Rayleigh interference optics
- UV-vis optics
- Sample cell (minus casing)
Relationship between data and sample

image from Ralston, 1993

https://www.beckman.com/resources/techniques-and-methods/analytical-ultracentrifugation
Sedimentation velocity (SV): shape & homogeneity

- Absorbance
- Radius
- Heterogeneity determination
- Sedimentation (s) & diffusion (D) coefficients (shape)
- Association/dissociation constant ($K_a/K_d$)
- Stoichiometry
- $M$ (from $s$ and $D$)

$t=0$

$t=1\ h$

$t=3\ h$
Sedimentation equilibrium (SE): mass and self-association

- Absorbance
- Radius

$t=0$
$t=1\ h$
$t=3\ h$
$t\approx24\ h+$

$M$ association/dissociation constant ($K_a/K_d$)
stoichiometry
non-ideality ($B$)
Outline

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<table>
<thead>
<tr>
<th></th>
<th>Absorbance</th>
<th>Interference</th>
<th>Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lowest conc</td>
<td>$A_\lambda = 0.1$</td>
<td>0.05 mg/ml</td>
<td>100 pM fluorescein</td>
</tr>
<tr>
<td>Dynamic range</td>
<td>2-3 logs</td>
<td>3-4 logs</td>
<td>6-8 logs</td>
</tr>
<tr>
<td>Radial res’n (µm)</td>
<td>20-50</td>
<td>10</td>
<td>20-50</td>
</tr>
<tr>
<td>Scan time (s/cell)</td>
<td>$\approx 60$</td>
<td>1</td>
<td>$\approx 15$</td>
</tr>
<tr>
<td>Utility</td>
<td>• Selectivity • Sensitivity • Non-dialysables</td>
<td>• Buffer absorbs, sample doesn’t • Variable extinction coefficient • Short column equilibrium • Rapid sedimenters</td>
<td>• Selectivity • Sensitivity • Non-dialysables • Limited sample</td>
</tr>
</tbody>
</table>

Adapted from a slide by Tom Laue
Sample preparation

- Purify by gel filtration or similar
  - Unless you want to know what is in the solution in its entirety
- Estimate concentration
  - Using e.g. NanoDrop
- (Dialyse sample against the desired solvent)
  - Possible problems with detergents
  - Required for interference optics only
- Choose windows
  - Sapphire windows
    - Necessary for interference optics
    - Good for all AUC optics
  - Quartz windows
    - No good for interference
Sample requirements

- Sample volume
  - SV
    - 360 µl (12 mm p.l.)
    - 90 µl (3 mm p.l.)
  - SE
    - 80 µl (2- or 6-channel)
    - 20 µl (8-channel - interference only)

- Sample concentration
  - Absorbance: $A\lambda \approx 0.1-1.0$ (12 mm p.l.)
    - $\lambda = 180-800$ nm
  - Interference: 0.05-30 mg/ml

- Sample reference
  - Absorbance: can be column eluant; dialysate better
  - Interference: must be dialysate

- Typical multiplexing: 3 or 7 sample holders / run
  - $\leq 28$ samples / run
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Which speed for SE?

SE: data recorded until no change...
confirmed by WINMATCH
SE: more than just mass
Self-association: “deconvolution” into individual components

experimental data = sum of species

- monomer
- dimer
- tetramer

absorbance vs. radius (cm)
Self association: SE data are the sum of exponentials

\[ A_r = \exp[\ln A_0 + H.M(r^2 - r_0^2)] + \exp[n_2 \ln A_0 + \ln K_a_2 + n_2 H.M(r^2 - r_0^2)] + \exp[n_3 \ln A_0 + \ln K_a_3 + n_3 H.M(r^2 - r_0^2)] + \exp[n_4 \ln A_0 + \ln K_a_4 + n_4 H.M(r^2 - r_0^2)] + E \]
Self-association: best model revealed by residuals
SEDPHAT: species analysis: monomer + heavy

$\chi^2 = 17$
SEDPHAT: species analysis: monomer + “dimer” (both fixed) + heavy

$\chi^2 = 8$
SEDPHAT: monomer-dimer equilibrium, $K_d = 10.8 \ \mu M$

$\chi^2 = 144$
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SV: radial movement recorded as function of time
Interference optics acquire refractive index data rapidly, independent of chromophores

hemocyanin sedimentation observed with interference optics
The Lamm equation describes SV

\[ \frac{\partial c}{\partial t} = -\frac{1}{r} \frac{\partial}{\partial r} \left( r \cos \theta \frac{\partial}{\partial r} \right) - D \frac{\partial^2 c}{\partial r^2} \]

\[ s = \frac{v}{\omega^2 r} \]

\[ s = \frac{M(1 - \nu \rho)}{N_A f} \]

- s is particle velocity per unit centrifugal field
- How s relates to mass and friction
s is influenced by solvent density & viscosity and sample partial specific volume (psv or vbar)

\[
s_{20,\text{w}} = \frac{(1 - \bar{v}\rho)_{20,\text{w}}}{(1 - \bar{v}\rho)_{\text{T,b}}} \frac{\eta_{\text{T,b}}}{\eta_{20,\text{w}}} = s_{\text{T,b}}
\]

- Sample partial specific volume ~ 1/density
- Sedimentation coefficient standardised to solvent of water @ 20°C
- \( \bar{v}\rho \) ~ 1.5 for typical aqueous solvent at 4°C
- Experimental sedimentation coefficient determined in e.g. buffer (b) at T°C
SEDNTERP: Calculation of $\rho$, $\eta$ & $psv$

http://rasmb.org/sednterp/
SEDFIT c(s) analysis: how many species + s of species

1: Load SV data

http://www.analyticalultracentrifugation.com/default.htm
2: Specify parameters

http://www.analyticalultracentrifugation.com/default.htm
3: Set meniscus, cell base and analysis limits

http://www.analyticalultracentrifugation.com/default.htm
4: Run

http://www.analyticalultracentrifugation.com/default.htm
5: Subtract time and radial invariant noise

http://www.analyticalultracentrifugation.com/default.htm
6: Fit (with solutions to the Lamm equation)

http://www.analyticalultracentrifugation.com/default.htm
7: Integrate to obtain estimate of concentration of species & weight-average values

http://www.analyticalultracentrifugation.com/default.htm
Two-dimensional spectrum analysis (2DSA) of SV data with UltraScan: model independent fitting giving $s$ & $M$

- Important when $f/f_0$ varies for components

Interacting systems & monodispersity

- **Rapid monomer-dimer**
  - SV will show 1 symmetrical boundary
  - Can be confused with monodispersity
  - Position will correspond to average of $s_{\text{monomer}}$ & $s_{\text{dimer}}$

- **Slow monomer-dimer**
  - Indistinguishable from mixture of monomer and dimer
    - Therefore 2 peaks / asymmetric single peak
    - Except if proportion of species depends on loading concentration
Life-time of FAM-GluA2 ATD dimer is significantly longer than that of Dylight488- or EGFP-GluA2 ATD

\[ K_D = 20.5 \text{ nM} \]
\[ s_1 = 3.52 \text{ S}, s_2 = 5.21 \text{ S} \]

\[ K_D = 25.4 \text{ nM} \]
\[ s_1 = 4.26 \text{ S}, s_2 = 6.44 \text{ S} \]

\[ K_D = 2.3 \text{ nM} \]
\[ s_1 = 3.52 \text{ S}, s_2 = 5.04 \text{ S} \]
Sedimentation velocity of proteins solubilised in detergent
SV of AcrB in DDM

- $s \approx 14.9 \, S$ for 66% of material (protein-detergent)
- $s \approx 3.3 \, S$ (observed from $\Delta J$) (micelles)

Ebel, Methods (2011) doi: 10.1016/j.ymeth.2010.11.00
Detergent solubilised proteins: density matching SE

- In SE buoyant molecular weight is determined:

\[ M_p(1 - \phi' \rho) = M_p[(1 - \bar{v}_p \rho) + \delta_{\text{Det}}(1 - \bar{v}_{\text{Det}} \rho)] \]

- In many AUC expts we want to observe self-association
- Density matching is a good method for self-associating membrane proteins

Density matching SE: experimental conditions

- Experimental conditions adjusted such that:
  - solvent $\rho = \text{effective } \rho$ of bound detergent

  $\rho = \frac{1}{\bar{v}_{\text{Det}}}$

- So detergent becomes effectively invisible to centrifugal field
- SE data can be analysed with standard methods
Determination of density-matching point for C14SB

- Determine % of D₂O required to density match C14SB micelles in background of other buffer components
  - 30 mM C14SB in 20 mM Tris-HCl, 200 mM KCl made in 0, 10, 20, 30% D₂O
  - Reference solvent the same minus detergent
  - SE observed with interference optics
    - Collect “buffer blanks” for subtraction to reduce noise
    - Then replace buffer with micelle solution in sample channel
    - Rotor speed 50k rpm
    - T = 25°C

Determination of density-matching point for C14SB

\[ \rho_{\text{micelle}} > \rho_{\text{solvent}} \]
\[ \rho_{\text{micelle}} = \rho_{\text{solvent}} \]
\[ \rho_{\text{micelle}} < \rho_{\text{solvent}} \]

Figure: Graph showing fringes versus radius and slope of distribution versus % D$_2$O.

SE of systems solublised by C14SB: OmpF

- E. coli OmpF
- Beckman XL-A, T = 25°C
- 20 mM Tris-HCl, 100 or 200 mM KCl
- 13% D₂O
- OmpF normally trimer
- Collected 36 data sets
  - [OmpF] = 0.3, 0.6, 0.9 A₂₃₀ (12 mm pathlength)
  - [C14SB] = 5, 12 & 30 mM
  - Rotor speed = 9, 11, 13.5, 16.3k rpm

OmpF

- Self-association probed in 2 ways:
  - Working at low [protein]
  - Increasing [detergent]
- At each [detergent], SE data globally fitted
  - For 4 rotor speeds & 3 [protein]

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s: deviation from sphericity + hydrodynamic hydration

\[ s = \frac{M(1-v_\rho)}{N_A f} \]

\[ M, f>f_0 \]

\[ M, f>f_0 \]

\[ M, f>>f_0 \]

\[ M, f>>f_0 \]
Sedimentation coefficient is a constraint for SAS modelling

- For one sphere
- For an assembly of N spheres
- where

\[ f_0 = 6\pi \eta_0 \sigma \]

\[ F_i = -f_i (u_i - v_i^0) - f_i \sum_{j=1}^{N} T_{ij} F_j \]

\[ T_{ij} = \frac{1}{8\pi \eta_0 R_{ij}} \left( I + \frac{R_{ij} R_{ij}}{R_{ij}^2} \right) \]

Different HM methods have their own pros & cons

Several freely available programs for HM

José García de la Torre et al.

- [http://leonardo.inf.um.es/macromol/programs/programs.htm](http://leonardo.inf.um.es/macromol/programs/programs.htm)
- HYDRO++
  - Computes hydrodynamic & other parameters for any bead model
- HYDROPRO
  - Computes hydrodynamic & other parameters for models constructed from pdb files
- And many other programs....

See also Rocco & Byron, Methods in Enzymology (2015) 562, 81-108
Several freely available programs for HM

- Mattia Rocco, Emre Brookes
  - http://somo.aucsolutions.com/
  - US-SOMO
    - AtoB and SOMO
      - Generates HBMs from pdb files, computes hydrodynamic & other parameters with “realistic” hydration
    - BEST (Sergio Aragon)
      - Boundary element modeling – very precise, computationally intensive
  - Zeno
    - Electrostatic-hydrodynamic analogy

See also Rocco & Byron, Methods in Enzymology (2015) 562, 81-108
SOMO is a subprogram of UltraScan III

Mattia Rocco/ Borries Demeler/ Emre Brooks


http://somo.aucsolutions.com/
SEC-SAXS and $c(s)$ of AUC SV are mirror images
SEC-SAXS and \( c(s) \) of AUC SV are mirror images
Select from Simulation drop-down menu

http://somo.aucolutions.com/
Load PDB file

http://somo.aucsolutions.com/
File read, checked for compatibility, displayed with RasMol

http://somo.aucosolutions.com/
Compute & display bead model

There are 3351 atoms in 1 chain(s) in this model
Creating beads from atomic model
Computing ASA via ASAB1
Return from Computing ASA
Anhydrnous volume 59506.41 A^3
There are 864 beads in this model before popping
Begin popping stage 1
Beads popped 0:
Begin radial reduction stage 1
Begin popping stage 2
Beads popped 0:
Begin radial reduction stage 2
Begin popping stage 3
Beads popped 0:
Begin radial reduction stage 3
Finished with popping and radial reduction
Rechecking beads
3 previously buried beads are exposed by rechecking
Finished rechecking beads
Build bead model completed
Visualizing model 1

http://somo.aucssolutions.com/
Compute & display hydrodynamic parameters

Rechecking beads
3 previously buried beads are exposed by rechecking
Finished rechecking beads
Build bead model completed
Visualizing model 1
Non-default options:
SDMO Options -> SoMo Overlap Reduction -> Bead Overlap Tolerance: 0.002

To reset to default: Menu bar -> Configuration -> Reset to Default Config

Begin hydrodynamic calculations
Model 1 will be included

Processing model 1 bead count 864 vbar 0.743
Using 260 beads for the matrix
Supermatrix inversion Cycle 1 of 3
Supermatrix inversion Cycle 2 of 3
Supermatrix inversion Cycle 3 of 3
Calculate hydrodynamics completed

http://somo.aucolutions.com/
Can also upload DAMs to SOMO

http://somo.aucssolutions.com/
Input psv and M for model

http://somo.aucssolutions.com/
Convert DAM to HBM

http://somo.aucsolutions.com/
Compute hydrodynamics for DAM-HBM: compare with experimental values

http://somo.aucsolutions.com/
Example: Oligomerisation of synthetic polyvalent integrin α5β1 ligands

- α5β1 ligands used to immobilise cells on surfaces via
  - 9th type III FN domain synergy site (**PHSRN**)
  - 10th type III FN domain **RGD** site

- α5β1 ligand oligomers facilitate increased binding

- Oligomerisation accomplished via 5 heptad repeats based on GCN4 leucine zipper
  - I/L placed variously at a and d positions to promote di-, tri- & tetramerisation

- Thiol-linked immobilisation to surface achieved via C-terminal Cys

- Question: do the ligands oligomerise as designed?
Construction of hydrodynamic bead models

- From vector (including His-tag) – too short for e.g. SWISSMODEL
- FN III 9-10 domain pair homology model (SWISSMODEL)
- Synthesised “missing beads”
- Coiled-coil (42 a.a.) – SWISSMODELs generated for underlined segment

Oligomerisation of synthetic polyvalent integrin $\alpha_5\beta_1$ ligands - AUC SV: c(s) reveals complex composition

"dimer"

"trimer"

"tetramer"

AUC SV + DTT: $c(s)$ composition simplified

Questions?

I MUSTACHE YOU

A QUESTION

Get More Funny Stuff @ funnyadudl.net

WHY THE HELL ISN'T THE IPHONE'S BATTERY LIFE CALLED

APPLE JUICE

If a dog wore pants would he wear them like this or like this?

If Apple made a car... would it have Windows!?

are you childish?

☐ yes
☐ no

What would your pro-wrestler name be?