SAXS studies on mixtures, assemblies, flexible systems

Melissa Gräwert
EMBL Hamburg
INTRO I

I(s)

Shape determination

Structure validation

Rigid body modelling

Missing fragments

Flexible systems

Oligomeric mixtures

INTRO I
Introduction:
* What mixtures are we talking about
* When can SEC-SAXS help?
* How do these influence the SAXS data

Case studies:
* Oligomeric equilibrium
  - Insulin, Sanofi
* Flexible systems
  * Higher molecular weight species
    - mAB, Roche
* Ensemble approach
- What mixtures are we talking about
  
  - Sample impurities: often unknown
Batch mode or SEC-SAXS mode

solubilized protein vs free micelles
aggregate vs monodisperse sample
oligomer vs monomer
complex vs subunits
X-ray scattering:
X-ray scattering:

A) Comparison of theoretical to experimental data

B) Transforming data into real space

C) *Ab initio* reconstruction

DAMMIN

DAMMIF

GASBOR

SASDBD — BSA Monomer, SASDF99

SASDBD — BSA Dimer, SASDFR8

MW$_\text{dimer}$ = 131 kDa

RG$_\text{dimer}$ = 4.1 nm

MW$_\text{monomer}$ = 60 kDa

RG$_\text{monomer}$ = 2.8 nm
Static & Dynamic Laser Light Scattering:

**X-ray scattering:**

\[
\begin{align*}
\text{MW}_{\text{dimer}} &= 132\text{kD} \\
\text{RH}_{\text{dimer}} &= 4.8\text{nm} \\
\text{MW}_{\text{monomer}} &= 64\text{kD} \\
\text{RH}_{\text{monomer}} &= 3.6\text{nm} \\
\text{MW}_{\text{dimer}} &= 131\text{k} \\
\text{RG}_{\text{dimer}} &= 4.1\text{nm} \\
\text{MW}_{\text{monomer}} &= 60\text{ kD} \\
\text{RG}_{\text{monomer}} &= 2.8\text{nm}
\end{align*}
\]
SEC-SAXS data processing

with CHROMIXS

CHROMIXS: automatic and interactive analysis of chromatography-coupled small-angle X-ray scattering data

Alejandro Panjkovich, Dmitri I Svergun


Published: 28 December 2017   Article history▼
Load data directory
Data from SASBDB : SASDFN8
Removal of pre-peak → separation

Strong signal

No background drift
Removal of pre-peak → separation
Strong signal
No background drift
Take all frames if possible

CHROMIXS
Sample Automatic Selection
Buffer Automatic Selection
Process Selection
Expected: 479 kD

~ 423 kD
Process Selection
- Batch mode or SEC-SAXS mode
Why not to perform SEC-SAXS(+MALLS)?
- Some practical examples
- Nice peak
- Removal from unbound substance
- Return to background
- Good data!
Why not to perform SEC-SAXS(+MALLS)?
- Some practical examples

Separation/Column interactions

Radiation damage
Why not to perform SEC-SAXS(+MALLS)?
- Some practical examples

**separation limits**

**Dilution effect**
Why not to perform SEC-SAXS(+MALLS)?
- There are some limits!

“Hmmm, I don’t know which peak it is?”
Why not to perform SEC-SAXS(+MALLS)?
- There are some limits!

1. Trip:  
   ![Graph 1]

2. Trip:  
   ![Graph 2]
When performing SEC-SAXS(+MALLS)?

Remember

- "ideal sample"
  - Pre-analysis of sample is very important

- not quite pure sample
  - SEC-SAXS is analytical! Not preparative!

- radiation damage can be a issue
  - Measure batch sample as well, add scavengers

- Sample stability, low affinity complexes
  - Sample can be altered with column interaction
What mixtures are we talking about

- Impurities in protein dilution: contaminations
- Oligomeric mixtures
- Assemblies (low affinity)
- Flexible systems
How do mixtures scatter?

In monodisperse systems (non-interacting):
- random orientation (tumbling in solution)
- size, shape and internal structure
How do mixtures scatter?

For equilibrium and non-equilibrium mixtures,
- scattering intensities of each species $I_k(s)$
- volume fractions $v_k$
How do mixtures scatter? And what if our system is flexible? **Conformational polydispersity (eg. IDPs)**

\[ I(s) = 4\pi \int_0^{D_{\text{max}}} p(r) \frac{\sin(sr)}{sr} dr \]

\[ I(s) = \sum_k v_k I_k(s) \]

- <Almost> infinite range of conformations
- Cannot really identify all possible \(v_k\) and \(I_k(s)\)
- Requires a more indirect approach
Introduction:

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* Oligomeric equilibrium
  (Insulin, Sanofi)

* Flexible systems
  * Higher molecular weight species
    (mAB, Roche)
  * Ensemble approach
    (characterization of a cancer drug target)

  (vaccine development)
1. Analysis of oligomeric species
MONOMER biologically active, circulating form *highly physicochemically unstable*

- **exogenous insulins** to ensure sufficient shelf life formulated as compact oligomers (mainly hexamers/crystalline state)

1. Analysis of oligomeric species

- fit (2VJZ.pdb)

Hexameric confirmation of the therapeutic insulin
MONOMER biologically active, circulating form

knowing the quaternary structure of insulin and insulin analogues under pharmaceutical formulation conditions is important for improving the physical and chemical stability of the drug product as well as for fine-tuning the absorption kinetics upon subcutaneous injection.

1. Analysis of oligomeric species
MONOMER biologically active, circulating form

Lantus®
Toujeo® → shift in isoel. point

Apidra®

1. Analysis of oligomeric species
Comparison: insulin glulisine in Apidra® insulin glargine in Lantus®

- Similar $I(0) = \text{similar in size}$
  $\rightarrow \text{MW estimates} \sim \text{hexamer}$

- Negative curvature at low $s$
  $\rightarrow \text{Repulsion more pronounced for insulin glargine}$

*Nagel et al. 2019 Biophys. Chem. 253:106226*
Comparison: insulin glulisine in Apidra® insulin glargine in Lantus®

- Similar I(0) = similar in size
  → MW estimates ~ hexamer

- Negative curvature at low s
  → Repulsion more pronounced for insulin glargine
  → prevent further association and undesired aggregation

- Faster decay of Apidra curve
  → larger species present in formulation

Nagel et al. 2019 Biophys. Chem. 253:106226
Insulin glulisine in Apidra® primarily consists of hexamers with a significant fraction of monomers and dodecamers which partially dissociate into monomers upon dilution with placebo.

- fits with dodecamers
- fits without dodecamers
Insulin glargine in Lantus® and Toujeo® show strong repulsive interactions under formulation conditions and strong attractive interactive upon dilution.

**Figure 5.** Scattering profiles of insulin glargine under formulation conditions and dilutions thereof. Scattering profiles of Toujeo® U300 (10.91 mg/ml (black)), Lantus® U100 (3.64 mg/ml (blue)), and Toujeo® at various dilutions in ddH₂O (c₀.₅ = 5.54 mg/ml, light gray, filled; c₀.₃₃ = 3.64 mg/ml, cyan, filled; c₀.₀₁ = 1 mg/ml, red) are displayed (A), on log scale and (B), on linear scale (for smallest angles).
In the formulation conditions of Lantus® and Toujeo® (insulin glargine), the drug substance seems to be in a more stable hexamer-dimer equilibrium with only trace amounts of dodecamers.


**Highlights**

- In marketed formulations, the two insulin analogues glulisine and glargine do not consist of pure hexamers.

- Insulin glulisine (Apidra) is predominantly present as hexamers with significant fractions of monomers and dodecamers under formulation conditions.

- Dilution studies, to characterize the association/dissociation properties and to mimic the subcutaneous scenario upon injection to some extent, reveal a dissociation of the hexamers and dodecamers into monomers for insulin glulisine.

- The insulin glargine in Lantus® and Toujeo® demonstrates a hexamer-dimer equilibrium, which is maintained upon dilution with water.

- SAXS powerful tool for such an investigation!

1. Analysis of oligomeric species
OLIGOMER

Computation of volume fractions of mixtures with known scattering intensities from the components
© ATSAS team 1994-2008

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119333 Moscow
Russia

- Manual
- Electronic reprint
- Questions and feedback
- Download
SASDF94 – Insulin glulisine (Apidra), oligomeric composition

Insulin glulisine

MW<sub>obs</sub> 34 kDa
MW<sub>expected</sub> 35 kDa
OLIGOMER
Form Factors for experimental data
C:/Users/graevert/Desktop/SASDF94/SASDF94.dat

Selected Form Factors (*.dat *.out *.pdb *.ent)
Form Factors for experimental data
C:/Users/graewert/Desktop/SASDF94/SASDF94.dat

Selected Form Factors (*.dat *.out *.pdb *.ent)

- SASDF94_fit1_model1.pdb
- SASDF94_fit1_model2.pdb
- SASDF94_fit1_model3.pdb
- SASDF94_fit1_model4.pdb

Next >
Computing the volume fractions, please be patient...

Form-factor number 4
Calculated MW and Rg  9447.012863   26.3157
Form-factor number 5
Calculated MW and Rg  29563.656743   0.0000
s range: .............................. 9.493E-03, 0.5034
Combinations =
  1    2    3
  4    5
Operable s range: .............................. 9.493E-03, 0.5001
Use non-negativity condition
Output file name: SASDF94.fit
>>> Processing complete!

OLIGOMER
OLIGOMER

Processing
Computing the volume fractions, please be patient ...

>>> 1/2

>>> ffmaker --undat 1 --unout 1 --lmax_max 20

>>> ffmaker --undat 1 --unout 1 --lmax_max 20 --sgrid SASDF94.dat SASDF94_fiti_model1.pdb SASDF94_fiti_model2.pdb SASDF94_fiti_model3.pdb SASDF94_fiti_model4.pdb

Calculating component 1 from 4

Read atoms and evaluate geometrical center ...

Number of atoms read ........................................ : 395
Number of atoms read ........................................ : 395

Geometric Center: 21.164 27.275 23.676

Percent processed 10 20 30 40 50 60 70 80 90 100

Processing atoms ............................................. : 254
Number of C ................................................. : 62
The image shows a computer screen with a statistical analysis software interface. The software displays a graph with data points and a table with test results.

### Test Results

<table>
<thead>
<tr>
<th>Test</th>
<th>Test Value</th>
<th>p-value</th>
<th>adj. p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CorMap Test</td>
<td>10</td>
<td>0.826339</td>
<td>0.826339</td>
</tr>
<tr>
<td>Red. χ² Test</td>
<td>1784</td>
<td>0.174600</td>
<td>0.174600</td>
</tr>
<tr>
<td>Anderson-Darling Test</td>
<td>0.347597</td>
<td>0.898609</td>
<td>0.898609</td>
</tr>
</tbody>
</table>
Files have not been saved.
Unsaved files will be removed from the file system.
Do you want to save the files before continuing?

Yes  No
Fit curve
Reconstruction of quaternary structure from X-ray scattering by equilibrium mixtures of biological macromolecules.

Petoukhov MV, Billas IM, Takacs M, Graewert MA, Moras D, Svergun DI

Author information

Biochemistry, 19 Sep 2013, 52(39):6844-6855
DOI: 10.1021/bi400731u  PMID: 24000896

Abstract

A recent renaissance in small-angle X-ray scattering (SAXS) made this technique a major tool for the low-resolution structural characterization of biological macromolecules in solution. The major limitation of existing methods for reconstructing 3D models from SAXS is imposed by the requirement of solute monodispersity. We present a novel approach that couples low-resolution 3D SAXS reconstruction with composition analysis of mixtures. The approach is applicable to polydisperse and difficult to purify systems, including weakly associated oligomers and transient complexes. Ab initio shape analysis is possible for symmetric homooligomers, whereas rigid body modeling is applied also to dissociating complexes when atomic structures of the individual subunits are available. In both approaches,
Direct shape determination of intermediates in evolving macromolecular solutions from small-angle scattering data

Petr V. Konarev a,b and Dmitri I. Svergun c,d

*Laboratory of Reflectometry and Small-angle Scattering, A. V. Shubnikov Institute of Crystallography of Federal Scientific Research Centre ‘Crystallography and Photonics’ of Russian Academy of Sciences, Leninsky pr. 59, Moscow 119333, Russian Federation, National Research Centre ‘Kurchatov Institute’, Akademika Kurchatova pl. 1, Moscow 123182, Russian Federation, and Hamburg Outstation, European Molecular Biology Laboratory, Notkestrasse 85, Hamburg 22607, Germany

*Correspondence e-mail: svergun@embl-hamburg.de
- **Kratky plot** ($I(s)s^2$ vs $s$)

This plot provides a sensitive means of monitoring the degree of compactness

- **Globular particle** : bell-shaped curve
- **Gaussian chain** : plateau at large $s$-values

!!! but beware: a plateau does not imply a Gaussian chain
- **Looking at flexible systems**

- **Kratky plot** \((I(s)*s^2 \text{ vs } s)\)
  
  This plot provides a sensitive means of monitoring the degree of compactness
  
  - Characterize proteins
  - Screen for changes

---

Mathiasen et al. 2016

---

**Flexible Systems**
Dimensionless Kratky plots

Characterization of mAb dimers reveals predominant dimer forms common in therapeutic mAbs. (Plath et al. 2016; Mabs)
Characterization of mAb dimers reveals predominant dimer forms common in therapeutic mAbs. (Plath et al. 2016; Mabs)

Summary:

- SAXS data supports TEM analyses and shape parameters from light scattering and AUC

- first study that extensively describes compact mAb species of dimer2

- The impact of these different dimer species on safety and efficacy is of great interest for future pharmaceutical development of therapeutic antibodies
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  (Insulin, Sanofi)
* Flexible systems
  * Higher molecular weight species
    (mAB, Roche)
  * Ensemble approach
    (characterization of a cancer drug target)
* (vaccine development)
Structural Characterization of Flexible Proteins Using Small-Angle X-ray Scattering

Pau Bernadó, Efstratios Mylonas, Maxim V. Petoukhov, Martin Blackledge, and Dmitri I. Svergun

View Author Information

Cite this: J. Am. Chem. Soc. 2007, 129, 17, 5656–5664
Publication Date: April 6, 2007
https://doi.org/10.1021/ja069124n
Copyright © 2007 American Chemical Society

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5344 4 750

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Ensemble approach

1) Pool of random structures are generated (~10,000) and their scattering profiles are calculated.

2) Ensemble of sub-structures are selected from this pool.

3) Compare distribution of structural parameters from group of substructures compared to those of complete pool.

Graewert & Svergun (2020) Biochem (Lond) 42(1), 36-42
Assessing overall flexibility

Conformational characterization of full-length X-chromosome-linked inhibitor of apoptosis protein (XIAP) through an integrated approach

Panagis Polykretis,a Enrico Luchinat,a,b Alessio Bonucci,a Andrea Giachetti,a Melissa A. Graewert,c Dmitri I. Svergunc and Lucia Banciad

aCTB—Magnetic Resonance Center, University of Florence, Via L. Sanguinetti 6, 50019 Sesto Fiorentino, Italy

Polykretis et al. 2019
Assessing overall flexibility

- X-chromosome-linked inhibitor of apoptosis protein (XIAP)
  - 497-residue cytoplasmic zinc-binding protein
  - expressed in most human tissues

- three zinc-binding baculovirus IAP repeat (BIR) domains
- a ubiquitin-associated (UBA) domain
- a C-terminal, zinc-binding Really Interesting New Gene (RING) domain

- XIAP = potent inhibitor of apoptosis (blocking the proteolytic activity of caspases)
- is overexpressed in tumours (potentiates cell survival and resistance to chemotherapeutics)

- XIAP has become an important target for the development of cancer treatments
Assessing overall flexibility

Ab initio modelling: disc-like flat conformation

SEC-SAXS:
Homomeric dimer
Rg 38 Å

$P(r)$:
almost Gaussian
→ compact

Dmax 130 Å

$R_g$: $\langle s(0) \rangle$ 

Polykretis et al. 2019
Assessing overall flexibility

- HADDOCK calculations (40,000 models)
- X-tal structures of single domains
- N-terminus and the inter-domain linkers make up more than 20% of the overall sequence
- preserve the BIR1 and RING homodimers (C2 symmetry)

- experimental restraints
  - SAXS: $R_g = 38\,\text{Å}$
  - EPR-DEER: $\text{Cys202–Cys202} = 38.6\,\text{Å}$
  - $\text{Cys351–Cys351} > 70\,\text{Å}$

Polykretis et al. 2019
Assessing overall flexibility

Crysol: Selection of HADDOCK models
Assessing overall flexibility

EOM:
Improved fit, selection of elongated compact structures
Assessing overall flexibility

- X-chromosome-linked inhibitor of apoptosis protein (XIAP)
- *first* structural model of the full-length XIAP dimer
- Integrated approach: nuclear magnetic resonance, small-angle X-ray scattering and electron paramagnetic resonance data
- XIAP adopts a compact and relatively rigid conformation, implying that the spatial arrangement of its domains must be taken into account when studying the interactions with its physiological partners and in developing effective inhibitors (not simplistic beads-on-a-string approach)
Utilization of Staphylococcal Immune Evasion Protein Sbi as a Novel Vaccine Adjuvant


1 Department of Biology and Biochemistry, University of Bath, Bath, United Kingdom, 2 Hamburg Unit, European Molecular Biology Laboratory, Deutsches Elektronen-Synchrotron, Hamburg, Germany, 3 Institute of Cellular Medicine, Newcastle University, Newcastle-upon-Tyne, United Kingdom, 4 Dynamic Biosensors GmbH, Martinsried, Germany, 5 Department of Chemistry, University of Bath, Bath, United Kingdom, 6 Department of Pharmacy and Pharmacology, University of Bath, Bath, United Kingdom
C3d-opsonised antigen complex

→ Co-ligation of the B cell antigen receptor with complement receptor 2 on B-cells

→ significantly lowers the threshold required for B cell activation

→ fusions of antigens with C3d polymers have shown great potential in vaccine design

→ unique complement activating characteristics *Staphylococcus aureus* immunomodulator Sbi to develop “pro-vaccines.”

→ spontaneously coats antigens with C3 degradation products in a natural way

*Yang et al. 2019*
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  * Ensemble approach
    (characterization of a cancer drug target)
* (vaccine development)
EOM online

Ensemble Optimization Method

Based on the provided peptide sequence and structured domains (if any) 10 000 random models will be generated. The experimental data will be fitted by an ensemble of 20 (or less) models.

Project description
- test

The first 5 characters in the description will be used to generate the project identifier.

Experimental data
- SASDA46.dat

Angular units
- 1/°

Overall symmetry
- P1 (no symmetry)

Sequence (*.seq etc.)
- test seq

Generate
- random coil models
- native-like models
- more compact models

Number of domains
- 2

Domain 1 (*.pdb)
- nterm.pdb
- monomer • free

Domain 2 (*.pdb)
- clerm.pdb
- monomer • free

SUBMIT

About EOM
Molecular basis of histone tail recognition by human TIP5 PHD finger and bromodomain of the chromatin remodeling complex NoRC.


Europe PMC

**SASDA46 – TIP5 in Tris**

Bromodomain adjacent to zinc finger domain protein 2A
> seq
KVTCLVCRKGDNDEFLLLCDGCDDRGCCHIYCHRPMCEAVPEGD
WFCTVCLAQQV
EGEFTQKPGFKRQKRKGYSLNFSGDGRRRVLLRGRE
SPAAGPRYSEEGLSPSKRRRLSRRNHQHSGSV
LTFCIEIILMEMESHDAAWPFLEPVNPRLSGYRRIIKNPMDFST
MRERLLRGGGYSSEEEFAADALLVFDNQCQTFMED
DSEVGKAGHIMRRFFESRWEFFY
**EOM online**

**Ensemble Optimization Method**

Based on the provided peptide sequence and structured domains (if any) 10 000 random models will be generated. The experimental data will be fitted by an ensemble of 20 (or less) models.

Project description: **test**

The first 5 characters in the description will be used to generate the project identifier.

**Experimental data**

Browse... SASDA46.dat

Angular units: 1/Å s = 4 msin(θ)/λ

Overall symmetry: P1 (no symmetry)

**Sequence (* seq etc.)**

Browse... test seq

Generate

- random coil models
- native-like models
- more compact models

Number of domains: 2

Domain 1 (*pdb)Browse... nterm.pdb monomer free

Domain 2 (*pdb)Browse... cterm.pdb monomer free

SUBMIT
Your job has been submitted...

You will be notified by email when the job is finished.

You may also bookmark this page to see the progress.

Thank you for using EOM.

---

test_494
  test_494_desc.txt
  nterm.pdb
  cterm.pdb
  test.seq
  SASDA46.dat
RANCH version 2.1 - r(12314)

Started: Mon May 25 16:42:04 2020
iSeed: -1136482726
Chain type: Random
Sequence file name: test.seq
Symmetry: p1
Symmetry type: aSymmetric
Number of residues per chain: 226
Number of atoms: 1330
Number of domains: 2

Domain number: 1
Path: nterm.pdb
Kept in the original PDB coordinates: 1
Oligomer: 0
DNA file name: None

Domain number: 2
Path: cterm.pdb
Kept in the original PDB coordinates: 0
Oligomer: 0
Dear ATSAS online user,

your EOM job test_494 is finished. The results are available here: 
https://www.embl-hamburg.de/biosaxs/atsas-online/eom.php?project=test_494

Thank you for using EOM.
Your ATSAS team
-- Chi^2 : 0.983

-- Files created:
Fit to the experimental data (in 1/angstrom) ........... : profiles_001_1.fit
Radius of gyration distribution (in angstrom) .......... : Rg_distr_001_1.txt
Max Dimensions distribution (in angstrom) ............. : Size_distr_001_1.txt
Ca(N)-Ca(C) distances distribution (in angstrom) ....... : CaCa_distr_001_1.txt
Volume distribution (in angstrom) ...................... : Volume_distr_001_1.txt

-- PDB models in the folder "GA001/curve_1/pdb":

<table>
<thead>
<tr>
<th>#</th>
<th>Filename</th>
<th>Rg</th>
<th>Dmax</th>
<th>Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>01895web.pdb</td>
<td>35.78</td>
<td>110.54</td>
<td>~0.22 (2/9)</td>
</tr>
<tr>
<td>2</td>
<td>04691web.pdb</td>
<td>27.04</td>
<td>88.32</td>
<td>~0.56 (5/9)</td>
</tr>
<tr>
<td>3</td>
<td>05221web.pdb</td>
<td>27.56</td>
<td>82.06</td>
<td>~0.22 (2/9)</td>
</tr>
</tbody>
</table>

Final ensemble : 29.10 91.87
Chi^2: 0.983

Files created:
- Fit to the experimental data (in 1/angstrom) : profiles_001_1.fit
- Radius of gyration distribution (in angstrom) : Rg_distr_001_1.txt
- Max Dimensions distribution (in angstrom) : Size_distr_001_1.txt
- Ca(N)-Ca(C) distances distribution (in angstrom) : CaCa_distr_001_1.txt
- Volume distribution (in angstrom) : Volume_distr_001_1.txt

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</tbody>
</table>

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  - Insulin, Sanofi
* Flexible systems
  * Higher molecular weight species
    - mAB, Roche
* Ensemble approach
Thank you: to everybody who contributed to all these programs

Thank you: to our Users with exciting biological questions (here: Sanofi, Banci Lab (Florence), Roche, van Elsen Lab (Bath))

Thank you: for your attention!