Hydrogen exchange mass spectrometry for higher-order structure determination in therapeutic protein discovery and development

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## Citations and links for this work

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<tr>
<th>Author(s)</th>
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Top 200 Pharmaceutical Products by US Retail Sales in 2012

Compiled and Produced by the Njardarson Group (The University of Arizona): Edon Vitaku, Elizabeth A. Hardi, Jón T. Njardarson

<table>
<thead>
<tr>
<th>Rank</th>
<th>Product</th>
<th>Company</th>
<th>2012 Sales</th>
<th>Category</th>
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<tbody>
<tr>
<td>6</td>
<td>Humira</td>
<td>AbbVie</td>
<td>$4,609 Million</td>
<td>SPEC ANTIRHEUMATIC AGENT</td>
</tr>
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49 biologics
Higher order structure is essential for function

Major Histocompatibility Complex (MHC) protein, http://www.youtube.com/watch?v=Y79XI0LfYI4
Applications of hydrogen exchange (HX)

Ligand binding

Protein interactions

Formulations

Disordered proteins

Epitope mapping

Comparability
Amide hydrogens serve as backbone sensors.

Leu  Ala  Pro  Lys  Ser

milliseconds to days

No exchange

Too fast
Hydrogen exchange reports on protein conformation and dynamics.

\[ k_{\text{obs}} = \frac{k_{\text{op}}}{k_{\text{cl}}} \times k_{\text{ch}} \]

- \( k_{\text{op}} \): Conformation and dynamics
- \( k_{\text{cl}} \): Chemical
H/D exchange kinetics probes backbone dynamics.

Flexible regions exchange rapidly
Rigid regions exchange slowly
MS approach uses quench and proteolysis.

- **Labeling** with D$_2$O
- **Quench** at 0 °C and pH 2.5
- **Proteolysis** using Pepsin
- **Mass Analysis**
Peptides progressively gain mass.
Efficient and robust platforms are now available
Applications of hydrogen exchange (HX)

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Disordered proteins

Epitope mapping

Comparability
Ricin as a vaccine target

- Extremely toxic (~1 µg/kg inhaled)
- Multiple organ failure
- Lethal within 72 hrs
- Potential terror agent
Vaccine discovery

1. Immunize animals
2. Antigen
3. Recover antibodies
4. Functional assays
5. Map the epitope

a. Map the epitope
b. Functional assays

Eliciting *neutralizing* antibodies

- Location?
- Mechanism?
- Rational design?
RTA peptic peptide map gives 100% coverage
Epitope mapping with hydrogen exchange

$1 - 12: \text{AIFPKQYPIINF}$

$94 - 108: \text{FHPDNQEDAEAITHL}$

$\Delta m = m_{\text{bound}} - m_{\text{free}}$
Epitope mapping by HX protection

Peptide Number

Relative Mass Difference (Da)

10 s

10^2 s

10^3 s

10^4 s

24 hr
Location of PB10 epitope by HX-MS
HX-MS agrees with Pepscan
Applications of hydrogen exchange (HX)

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Epitope mapping

Comparability

- Y
- Y
- Y
- Y
- Y
- X
mAbs are the Cadillacs of biotherapeutics.

- 150 kDa
- IgG1
- Glycosylated
- 12 disulfide bonds
- 50 mg/mL, pH 6
Large dose, small volume

Pre-filled syringe

40 mg mAb
0.8 mL

Plunger Rod
Finger Grip
Needle Cover
Reversible self-association

40 mg in 0.8 mL

Viscosity
Loss of efficacy
Immunogenicity
mAb C undergoes reversible self-association

Promoted by high pH and sulfate
Sulfate promotes association

![Graph showing the effect of sulfate on viscosity]

**Graph Explanation:**
- The graph illustrates the change in viscosity (cP) as a function of protein concentration (mg/mL) under different conditions.
- The x-axis represents protein concentration (mg/mL), while the y-axis represents viscosity (cP).
- Two conditions are highlighted:
  - 300 mM Na$_2$SO$_4$ at 4 °C
  - 300 mM NaCl at 4 °C

**Key Points:**
- Sulfate at 300 mM enhances the viscosity more significantly at 4 °C compared to the control conditions.
- The graph visually supports the statement that sulfate promotes association.
Mapping protected interface with HX-MS

- **mAb-C Heavy 11-22 (C_L)**
- **mAb-C Heavy 135-140 (C_H2)**
- **mAb-C Heavy 166-172 (V_H)**
- **mAb-C Heavy 45-59 (V_H)**
- **mAb-C Heavy 36-54 (V_L)**
- **mAb-C Heavy 48-70 (V_L)**

For each mass region, graphs show the mass increase (Da) over time (seconds) for two concentrations of mAb-C:
- 5 mg/mL (black line)
- 60 mg/mL (red line)

- **mAb-C (5 mg/mL)**
- **mAb-C (60 mg/mL)**
Mechanism of RSA

A

Significant increase
No Change
Significant decrease
No data available

CDR region

Histidine residues in CDR

B

C
Applications of hydrogen exchange (HX)

- Ligand binding
- Protein interactions
- Formulations
- Disordered proteins
- Epitope mapping
- Comparability

The diagram illustrates the applications of hydrogen exchange (HX) in various biological contexts. The illustrations show the relevance of HX in ligand binding, protein interactions, and formulating proteins. The disordered proteins, epitope mapping, and comparability are also depicted, highlighting the versatility of HX in scientific research.
Maintaining the physical stability of protein therapeutics is a critical problem.

Solution:
Develop a stabilizing formulation.
Rational formulation requires mechanistic understanding.
Does backbone flexibility correlate with stability?

- Thiocyanate
- Arginine
- Chloride
- Sucrose
- Sulfate
- mAb-B
- IgG1
- Thermal stability
- Aggregation
- Backbone dynamics
The connection between flexibility and stability was not obvious.

Loss of stability correlated with increased flexibility in $C_{H2}$ domain.
Sulfate slowed aggregation.
Thiocyanate accelerated aggregation.
Stabilizers and destabilizers acted as expected.

<table>
<thead>
<tr>
<th>Excipient</th>
<th>$\Delta T_m$</th>
<th>Aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>thiocyanate</td>
<td>$-9.0 , ^\circ C$</td>
<td>Faster (++)</td>
</tr>
<tr>
<td>arginine</td>
<td>$-1.9 , ^\circ C$</td>
<td>Faster (+)</td>
</tr>
<tr>
<td>chloride</td>
<td>$+0.3 , ^\circ C$</td>
<td>Negligible</td>
</tr>
<tr>
<td>sucrose</td>
<td>$+1.5 , ^\circ C$</td>
<td>Slower (−)</td>
</tr>
<tr>
<td>sulfate</td>
<td>$+1.8 , ^\circ C$</td>
<td>Slower (−)</td>
</tr>
</tbody>
</table>

How do these excipients work at the molecular level?
The effects of excipients are not uniform.

- Arg, 0.5 M
- NaCl, 0.1 M
- sucrose, 0.5 M
The effects are excipient-dependent.

Differential deuterium uptake (Da)

arginine, 0.5 M

sucrose, 0.5 M

120 s

$10^3$ s

$10^4$ s

$10^5$ s

Peptide (N to C)

±0.59 Da (99% CI)
The correlation between stability and altered H/D exchange is not obvious.

Homology model based on [Saphire, 2001] 1HZH
Extremes have nearly-identical effects.

SCN$^-$ Destabilizer

$\text{SO}_4^{2-}$ Stabilizer
The correlation with altered hydrogen exchange is not obvious.
Destabilizers have very different effects.
A hydrophobic segment of the $C_H^2$ domain may mediate aggregation.

Destabilizers and oxidation increased backbone flexibility. [Houde, 2010]

Protein A binding inhibits aggregation. [Zhang, 2012]

Disulfide bond increased thermal stability. [Gong, 2009]
Summary

Epitope mapping

Protein interactions

Formulations

ricin antibody

mAb self-association

aggregation hotspots
University of Kansas: David Volkin & Russ Middaugh
Wadsworth Center, NY State Dept. of Health: Nick Mantis
MedImmune: Hardeep Samra, Hasige Sathish, Reza Esfandiary, Steven Bishop, Prakash Manikwar