PROTEIN-PROTEIN INTERACTIONS, VISCOSITY AND INJECTABILITY IN MULTI PROTEIN CO-FORMULATIONS - BEVACIZUMAB AND rALBUMIN

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At Novozymes Biopharma A/S

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Case study – on aggregation prevention

• Analytical challenges and preliminary data

• Mechanism
  • SAXS
  • DLS
  • AF4
  • Viscosity
HUMAN SERUM ALBUMIN - PROPERTIES

- **Structure**
  - 585 amino acids, Single chain
  - Three domains
  - 17 disulfide bridges
  - **Cys34 is unpaired**
  - One tryptophan
  - 66472 g/mol
  - **Hydrophobic patches/cavities**
- IpH = 5.9
- **Soluble up to >400 g/L**
- Approximately 50 g/L in blood
- High physical stability
- Long plasma ½ life
  - 19-20 days
- Inert
  - Safe

Location of long chain fatty acid binding sites
Curry et al. (1999)
*BBA* **1441**, 131-140
How does rAlbumin stabilize protein formulations

<table>
<thead>
<tr>
<th>HSA in blood</th>
<th>rAlb in formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple hydrophobic binding sites</td>
<td>Coat hydrophobic surfaces in primary packaging materials</td>
</tr>
<tr>
<td>Increases the colloidal stability of blood</td>
<td>Prevent self-association of protein drugs</td>
</tr>
<tr>
<td>Natural antioxidant in blood</td>
<td>Prevent oxidation of protein drugs</td>
</tr>
</tbody>
</table>
ANALYSIS OF AGGREGATES
- A CHALLENGE FOR COMPLEX FORMULATIONS
- PRELIMINARY DATA
Prevention of protein aggregation in high concentration mAb formulations

Method set-up

Avastin (Bevacizumab) → Recombumin Alpha (rAlb) or buffer → Formulation

Transfer to Glass vial

29 days 40°C

AF4-UV-MALS-RI analysis
AF4-UV-MALS-RI ANALYSIS

- **AF4**: Asymmetric Flow Field Flow Fractionation
- **Separation takes place in the channel based on differences in diffusion**

AF4 separation works well with 4 out of 4 tested mAbs:
- **Omalizumab** (blue)
- **Rituximab** (green)
- **Tocilizumab** (red)
- **Bevacizumab** (next slide)
Aggregation prevention of ≥100 mg/mL Bevacizumab by 50 mg/mL rAlbumin

- AF4 separation:
  Short channel
  Spacer 350S
  10 kDa RC membrane
  50 mM NaNO₃, pH 7.2
  Channel flow 1 ml/min
  Cross flow gradient 3-1 mL/min (15 min)

- Bevacizumab:rAlb mixture ≥100:50 mg/mL (in red)
- Avastin 25 mg/mL (in blue)

<table>
<thead>
<tr>
<th></th>
<th>Monomer</th>
<th>Dimer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avastin</td>
<td>93,0</td>
<td>6,4</td>
</tr>
<tr>
<td>Bevacizumab+albumin</td>
<td>92,3</td>
<td>2,2</td>
</tr>
</tbody>
</table>

Confirmed:
- A1. Albumin monomer (66.4 kDa)
- A2. Albumin dimer (132.8 kDa)
- M1. Bevacizumab monomer (149 kDa)
- M2. Bevacizumab dimer (298 kDa)

Not yet confirmed but ration matches:
- FAB
- HC-HC
Prevention of freeze-thaw induced formation of sub-visible particles in proteins/peptides?

**Method set-up**

- RoActemra (Tocilizumab)
- Albucult (rAlb) or buffer

**Transfer to Eppendorff tube**

- 20°C 1 h
- -20°C >23 h

**Analysis by Micro-flow Imaging (MFI)**

Sub-visible particles (1-100 µm)
Albucult® prevents the formation of sub-visible particles in Tocilizumab (a monoclonal antibody)

Reference formulations
- 3.4 μm (0.5 mg/mL) Tocilizumab (diluted w/ milliQ)
- 137 μm (20 mg/mL) Tocilizumab (RoActemra®, Roche)
- 15 μm (1.0 mg/mL) Albucult (dil. w/ milliQ)
- 150 μm (10 mg/mL) Albucult (dil. w/ milliQ)

Test samples
- 0.5 mg/mL Tocilizumab formulated with Albucult in mAb:rAlb molar ratios
  - 1:1
  - 1:5
  - 1:10

ALBUCULT® PREVENTS FORMATION OF FREEZE THAW INDUCED SUB-VISIBLE PARTICLES IN MAB FORMULATIONS
Mechanism behind aggregation prevention
Small Angle X-ray Scattering Determination of solution structure


MaxLab Beamline I911-SAXS
Pair distribution function
rAlb is a monomer with a concentration dependent repulsive behaviour

- Repulsion evaluated from $I(0)$
- Repulsion evaluated from peak
No concentration dependent self-association of Bevacizumab (up to 30 mg/mL)
Co-formulation of Bevacizumab and rAlbumin

Bevacizumab flexibility decreases with increasing rAlbumin concentration

<table>
<thead>
<tr>
<th>BEV mg/ml</th>
<th>HSA mg/ml</th>
<th>MM (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.446</td>
<td>129.66</td>
</tr>
<tr>
<td>1</td>
<td>0.89</td>
<td>108.22</td>
</tr>
<tr>
<td>1</td>
<td>2.23</td>
<td>94.98</td>
</tr>
<tr>
<td>1</td>
<td>4.46</td>
<td>78.13</td>
</tr>
<tr>
<td>20</td>
<td>8.92</td>
<td>135.55</td>
</tr>
<tr>
<td>20</td>
<td>18</td>
<td>111.68</td>
</tr>
<tr>
<td>20</td>
<td>44.46</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>89.22</td>
<td>-</td>
</tr>
</tbody>
</table>
rAlbumin stabilizes Bevacizumab through molecular crowding

- curve fitting by linear combination
- Repulsive behavior of rAlbumin not perturbed by Bevacizumab
- No interaction between rAlb and mAb
Evaluation of mAb-rAlbumin protein-protein interactions by DLS

Method set-up

Avastin (Bevacizumab) → Recombumin Alpha (rAlb) → Formulation in different rAlb:mAb ratios → Mix → Wyatt DynaPro Plate Reader (DLS)
The protein-protein interaction is too weak to detect.

<table>
<thead>
<tr>
<th>Item</th>
<th>Mass Ratio</th>
<th>Radius (nm)</th>
<th>Expected Radius (nm)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 B8 Albucult</td>
<td>0</td>
<td>2.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 B9 Albucult</td>
<td>0</td>
<td>2.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19 B20 Avastin/Albucult 1:4</td>
<td>0.25</td>
<td>3.5</td>
<td>3.5</td>
<td>1.02</td>
</tr>
<tr>
<td>20 B21 Avastin/Albucult 1:4</td>
<td>0.25</td>
<td>3.5</td>
<td>3.5</td>
<td>1.00</td>
</tr>
<tr>
<td>21 B22 Avastin/Albucult 1:2</td>
<td>0.33</td>
<td>3.9</td>
<td>3.8</td>
<td>0.97</td>
</tr>
<tr>
<td>22 B23 Avastin/Albucult 1:2</td>
<td>0.33</td>
<td>3.8</td>
<td>3.8</td>
<td>0.98</td>
</tr>
<tr>
<td>23 C2 Avastin/Albucult 1:1</td>
<td>0.50</td>
<td>4.4</td>
<td>4.2</td>
<td>0.96</td>
</tr>
<tr>
<td>24 C3 Avastin/Albucult 1:1</td>
<td>0.50</td>
<td>4.5</td>
<td>4.2</td>
<td>0.93</td>
</tr>
<tr>
<td>11 B12 Avastin FB</td>
<td>1</td>
<td>5.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 B13 Avastin FB</td>
<td>1</td>
<td>5.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

• Observed radius of mixture comparable to expected radius of two non-interacting species.
VISCOSITY

- A CHALLENGE FOR PROTEINS IN HIGH CONCENTRATIONS
CONCENTRATED rALB FORMULATIONS PRESENT AN EXCELLENT INJECTABILITY* UP TO AT LEAST 300 MG/ML

- Viscosity and injectability are very well correlated properties for rAlb formulations
- The concentration of critical injectability is between 300 and 400 mg/mL where the viscosity markedly levels up
- Between 280 and 500 mg/mL, the viscosity of rAlb in solution increases exponentially with protein concentration

*30G ½”, 2 mL/min
THE INJECTABILITY* OF BEVACIZUMAB BECOMES CRITICAL BETWEEN 140 AND 180 MG/ML

- Viscosity and injectability are well correlated properties for Bevacizumab formulations.
- The concentration of critical injectability is between 140 and 180 mg/mL.
- This is in agreement with the viscosity increase between 150 and 230 mg/mL.
- Between 100 and 400 mg/mL the viscosity of Bevacizumab in solution increases exponentially with protein concentration.

*30G ½”, 2 mL/min
THE ADDITION OF rALB TO BEVACIZUMAB DOES NOT LEAD TO A SIGNIFICANT INCREASE OF THE INJECTION FORCE*

- The injection force of rAlb (50 mg/mL) is comparable to that of the buffer.
- At a total protein concentration of 150 mg/mL, the addition of rAlb leads to a minor increase of the injection force.
- At a total protein concentration of 250 mg/mL, it seems that rAlb eases the injection of Bevacizumab to a measurable value.

*30G ½”, 2 mL/min
MAJOR CONCLUSIONS

Rheology and viscosity
• The viscosity of rAlb formulations is comparable to buffer solutions up to more than 100 mg/mL
• An exponential increase in viscosity and injection force is observed for rAlb and two mAbs at high concentrations
• Adding rAlb to high concentration mAb formulations does not give an exponential increase in injection force

Analytics
• Af4 (FFF) is applicable to mixed formulations of mAbs and rAlb
• Combining IEX and SEC is a feasible method for analysis of 4 out of 5 mAbs separating mAbs and rAlb

Safety
• GMP manufacturing in yeast (animal free)
• Phase I clinical trial performed with no adverse events (50 /dose IV and 65 mg/dos IM)
• Used in marketed products

Aggregation prevention
• Recombumin significantly reduces heat and freeze thaw induced particle formation in formulations of mAbs

Mechanism behind aggregation prevention (one case study)
• Repulsive behavior and crowding effect as determined by SAXS and confirmed by DLS, AF4 and Rheological studies
Acknowledgements

SAXS analysis are performed in collaboration with DTU Chemistry, Technical University of Denmark

- Professor Pernille Harris
- Ph.D. stud Pernille Sønderby

Small Angle X-ray scattering (SAXS) data collection was performed at MAX-IV Beam-line I-911-4

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- Corinne Eenschooten
- Mette Larsen
- Anne Marie Scharff-Poulsen
- Stina Engelhardt
- Mikael Bjerg Caspersen
- Paul Luigi Gargani Weisbjerg

The organizing committee

- For organizing such fine an event
- For giving me the opportunity to present

DLS analysis are performed in collaboration with Wyatt Technology

- Roger Scherrers

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Strategy

- Separate albumin from mAb by in-line column capture of albumin under conditions where mAb elution is not affected.
- When mAb has sufficiently eluted, a high salt (or pH shift) is applied to elute albumin from the capture column.
- mAb aggregation can be analyzed, while albumin is either captured or undergoing elution.

Column system

- One anion exchanger with minimal void volume (capture column).
- One size exclusion column.
mAb AGGREGATION SUCCESSFULLY ASSESSED BY NEW IEX-SEC METHOD

**Materials**
- mAbX/albumin mixture (1:1 w/w)

**Method**
- Mobile phase A (pH 7.5): Tris (25 mM), NaCl (0.2 M)
- Mobile phase B (pH 7.5): Tris (25 mM), NaCl (1.0 M)
- Anion exchanger: ProSwift SAX-1S 4.6 x 50 mm
- Size exclusion column: TSK G3000 SWXL 7.8 x 300 mm

**Results**
- Baseline separation between the mAb and albumin can only be achieved when using the new IEX-SEC method
The new IEX-SEC method was successfully applied to a range of mAbs and mAb/albumin weight ratios.

<table>
<thead>
<tr>
<th>mAb tested</th>
<th>IpH</th>
<th>Min. weight ratio for successful separation</th>
<th>Max. advised albumin weight ratio for formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb X</td>
<td>5.8</td>
<td>1:100</td>
<td>100 x</td>
</tr>
<tr>
<td>Omalizumab</td>
<td>8.3</td>
<td>1:30</td>
<td>30 x</td>
</tr>
<tr>
<td>Bevacizumab</td>
<td>10</td>
<td>1:5</td>
<td>5 x</td>
</tr>
<tr>
<td>Tocilizumab</td>
<td>&gt;10</td>
<td>N/A*</td>
<td>N/A</td>
</tr>
</tbody>
</table>

The size exclusion column is not operable at high pH. When pH is lower than IpH, the positively-charged mAbs interact with the size exclusion column (cation exchanger) which hampers the analysis.