Protein Aggregates and Subvisible Particles
What are they and what is their clinical impact?

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All protein therapeutics contain (higher or lower levels of) aggregates and particles.

Most of these products are immunogenic.

Is there a link???
When does protein aggregation occur?

**Processing steps**

- Fermentation/Expression
- Purification
- Formulation
- Filling

**Handling and storage of final product**

- Transport
- Storage
- Administration

Adapted from Vasco Filipe
Some of the factors influencing protein aggregation

**Environmental factors**
- Temperature
- Interfaces
- Freeze-thaw
- Container

**Solution factors (formulation)**
- pH
- Excipients
- Ionic strength
- Concentration

Adapted from Vasco Filipe
Protein aggregates: definition and categories

- Protein aggregates = assemblies of protein molecules
- Protein aggregates are heterogeneous regarding (Narhi et al., J Pharm Sci 101, 493-498):
  - Size
  - Reversibility
  - Protein conformation
  - Covalent modification
  - Morphology
- So, the question “which aggregates do matter?” is difficult to answer
Example: even a simple aggregate such as a dimer can adopt various shapes and characteristics.

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<table>
<thead>
<tr>
<th></th>
<th>monomer</th>
<th>process stress dimer</th>
<th>pH stress dimer</th>
<th>light stress dimer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Covalent Aggreg.</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>Hydrophobicity</td>
<td>low</td>
<td>low</td>
<td>high</td>
<td>heterogeneous</td>
</tr>
<tr>
<td>Relative Potency</td>
<td>100%</td>
<td>62 ± 3%</td>
<td>101 ± 4%</td>
<td>21 ± 17%</td>
</tr>
</tbody>
</table>
```

Definitions by size (all are aggregates)

- **Oligomers**: < 100 nm
- **Particles**
  - **Submicron**: 0.1 – 1 µm
  - **Micron**: 1 – 100 µm
  - **Visible**: ≥ 100 µm

Size categories

Adapted from Linda Narhi
Complementary analytics needed to cover the size range

- Visual inspection
- Microscope
- Light Obscuration
- Micro Flow Imaging
- Field Flow Fractionation
- Nanoparticle Tracking Analysis
- Dynamic Light Scattering
- SDS-PAGE
- HP-SEC

Size range:
- 1 nm
- 10 nm
- 100 nm
- 1 µm
- 10 µm
- 100 µm
- 1 mm

Adapted from Michael Wiggenhorn, Coriolis

- MONOMERS
- OLIGOMERS
- SUBMICRON
- MICRON
- VISIBLE
Size-exclusion chromatography results do not predict particle levels.

Batch X
Optically clear
\[ \text{OD}_{350} = 0.002 \]

Batch Y
Visible aggregates
\[ \text{OD}_{350} = 0.1 \]
Submicron-sized particle counts do not predict micron-sized particle counts, vice versa.

Nanoparticle tracking analysis (submicron particles)

Light obscuration (micron particles)

Results of monoclonal human IgG1 stressed under different conditions

Micron-sized IgG aggregates induced by shaking remain at SC injection site for longer than a month

Filipe et al, manuscript in preparation
Our vaccines are based on particles in the ‘gap’ range

<table>
<thead>
<tr>
<th>Vaccine category</th>
<th>Examples</th>
<th>Particle category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live bacteria</td>
<td>Salmonella</td>
<td>Micron (~ 1 µm)</td>
</tr>
<tr>
<td>Inactivated bacteria</td>
<td>Whole cell pertussis</td>
<td>Micron (~ 1 µm)</td>
</tr>
<tr>
<td>Live viruses</td>
<td>Oral polio</td>
<td>Submicron (~ 30-300 nm)</td>
</tr>
<tr>
<td></td>
<td>Measles-mumps-rubella</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nasal influenza</td>
<td></td>
</tr>
<tr>
<td>Inactivated viruses</td>
<td>Inactivated polio vaccine</td>
<td>&lt; 100 nm</td>
</tr>
<tr>
<td></td>
<td>Whole inactivated influenza vaccine</td>
<td>Submicron (~ 200 nm)</td>
</tr>
<tr>
<td>Virus like particles</td>
<td>Hepatitis B</td>
<td>&lt; 100 nm</td>
</tr>
<tr>
<td>Split- and subunit vaccines</td>
<td>Influenza</td>
<td>Submicron (aggregates, ~ 300 nm)</td>
</tr>
<tr>
<td>Alum-adsorbed antigens</td>
<td>Diphtheria, tetanus</td>
<td>Micron (low µm range)</td>
</tr>
<tr>
<td>MF59 adjuvanted antigens</td>
<td>Influenza</td>
<td>Submicron (~200 nm)</td>
</tr>
</tbody>
</table>
Our vaccines are based on particles in the ‘gap’ range

Bachmann & Jennings, Nature Reviews 2010
New insights from quantifying subvisible particles

- Beyond potential immunogenicity, particle sizes and levels are important product quality attributes

- Mass of protein (e.g., < 0.1%) in particles may not be detectable as loss of monomer

- Subvisible particle analysis provides very sensitive early detection of protein aggregation and new insights into aggregation pathways, manufacturing & formulation development

- Even trace levels of particles can impact subsequent stability of protein solutions (see recent papers Carpenter and colleagues)
Beyond protein particles...  
Nonproteinaceous particles (often in the ‘gap’ range)

- **Glass** particles from containers
- Glass cartridges and syringes are siliconized, and free **silicone oil** droplets can be generated
- In syringes, there may be **tungsten** particles and salts from needle insertion process
- **Rubber or silicone** particles can come from stoppers
- **Stainless steel** and other particles from filling pumps
- **Particles shed from filters** during pre-filling sterile filtration

Protein molecules can adsorb to these particles  
This will create a “vaccine”
Subvisible particles and immunogenicity

Subvisible particles are in every therapeutic protein product and most of these products are immunogenic

Is there a link???

EXAMPLES
Aggregate/particle removal can induce tolerance

Already known in the 1960s!

Administration of aggregate-free foreign protein induces immunological tolerance in animals and human patients

For instance:
Dresser, *Immunology* 5, 378 (1962)
Biro & Garcia, *Immunology* 8, 411-419 (1965)
Spiegelberg & Weigle, *Int Arch Allergy* 31, 559-567 (1967)
Von Felten & Weigle, *Cellular Immunology* 18, 31-40 (1975)
Fujiwara et al., *Jpn J Microbiol* 20, 141-146 (1976)
Aggregate/particle removal can induce tolerance

Already known in the 1960s!

- Anti-human lymphocyte IgG produced in horses
- Administration to organ transplant patients resulted in antibody response and consequently rapid drug clearance
- Administration of equine IgG in which aggregates / particles were removed by ultracentrifugation (133,500 x g for 1 h) resulted in no immune response and made the patients tolerant for equine IgG
- Co-medication: azathioprine + prednisolone

Commercial beta-interferon products: immunogenicity in patients

- Percentages of patients forming binding antibodies and neutralizing antibodies in various clinical studies (measured with various assays)

### Submicron particle counts (NTA)

- Betaseron: 3 x 10⁸ Particles/mL (100nm-450nm)
- Extavia: 2 x 10⁸ Particles/mL (100nm-450nm)
- Rebif: 1 x 10⁸ Particles/mL (100nm-450nm)
- Avonex: 1.5 x 10⁸ Particles/mL (100nm-450nm)

*Barnard et al., J Pharm Sci 102: 915-928 (2013)*

### Neutralizing antibodies

- **Betaferon**
  - % positive patients: 47%, 28%, 14%
- **Rebif**
  - % positive patients: 38%, 5%
- **Avonex**
  - % positive patients: 14%, 2%

*Van Beers et al., J Interferon Cytokine Res 30: 767-775 (2010)*
rhIFNβ-1a with different aggregate levels

1. Bulk rhIFNβ-1a
   - Received as frozen bulk
   - In PBS (!) pH 7.2

2. Reformulated rhIFNβ-1a
   - Filtered and dialyzed bulk
   - In NaAc pH 4.8
   - Formulated with Tween 20 and ArgHCl

3. Stressed rhIFNβ-1a
   - Bulk incubated at pH 2 + 1 M NaCl
   - Aggregates purified by SEC-HPLC
   - In PBS pH 7.2

Subvisible particle counts and immunogenicity

- Hardly any particles in reformulated rhIFNβ-1a
- Immunogenicity in transgenic immune tolerant mice correlates with subvisible particle counts (rather than total % aggregates)

Adsorption of rhIFNβ to metal (but not glass or polystyrene) beads enhances its immunogenicity in transgenic mice.

Anti-rhIFNβ antibody titers after a 3-week injection protocol:

- **Metal**: Ø 14 μm, 71% adsorbed
- **Glass**: Ø 1.1 μm, 78% adsorbed
- **Polystyrene**: Ø 0.21 μm, 46% adsorbed

Adsorption of rhIFNβ to metal (but not glass or polystyrene) beads enhances its immunogenicity in transgenic mice

Anti-rhIFNβ antibody titers after a 3-week injection protocol

Subvisible particles break immune tolerance in mice to murine growth hormone

![Graph showing the relative anti-mGH (ng/ml) levels for different particles and doses.]

- Stock (20 ng): 6/8
- High-pressure treated stock (0.02 ng): 0/8
- Alhydrogel (2 ug): 8/8
- Glass (2 ug): 8/8

**Particles (dose):**

- IgG3
- IgG2c
- IgG2b
- IgG2a
- IgG1

**Relative anti-mGH (ng/ml):**

- 14000
- 12000
- 10000
- 8000
- 6000
- 4000
- 2000
- 0

**Courtesy of John F Carpenter**

Fradkin et al, J Pharm Sci 100, 4953-4964 (2011)
Conclusions

• Aggregates, including subvisible particles, are critical quality attributes

• Removal of aggregates & particles reduces protein immunogenicity

• Betaferon contains large amounts of aggregates & particles and is the most immunogenic rhIFNβ product

• Adsorption of protein to non-proteinaceous subvisible particles may increase immunogenicity risk

• However, no general rules: rhIFNβ adsorbed to glass particles was not very immunogenic, whereas mGH adsorbed to the same glass particles was
so the picture is not yet totally clear...

.....we are only at the beginning of our understanding about the relationship between protein aggregation, particle formation and immunogenicity
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