A novel flow cytometry based technology for the quantification and characterization of sub-visible particles in protein therapeutics

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Aggregation is a generic property of a polypeptide chain

Jahn & Radford, FEBS Journal, 2005, 75
“Undissolved species (other than gas bubbles or droplets) that are unintentionally present in the product. Particles can be foreign (not intrinsic to drug substance) or protein-related (i.e. large aggregates). Particles can be further categorized as visible (>ca. 50 µm) and sub-visible (between ca. 0.1–50 µm); submicron particles (between ca. 0.1–1 µm) are a subcategory of sub-visible particles.”

European Immunogenicity Platform (EIP), the Protein Characterization Subcommittee (EIP-PCS)

den Engelsman et al Pharm Res. 2011 Apr;28(4):920
Methods for size determination of sub-visible particles and aggregates

Tiede et al, Food Additives and Contaminants, 2008, 25
Flow cytometry based sub-visible particle detection and analysis

Flow cytometry based sub-visible particle detection and analysis

Not detectable | Non-protein particles; detected | Protein particles; detected and characterized

| < 100 nm | (0.1 µm) 0.75 µm – ca 70 µm |

- Particles larger than 100 nm can be detected
- Detected particles can be counted
- The size and properties of particles can be determined

Protein specific dye | Protein monomer | Protein particles | Non-protein particle
Attribute specific dye
<table>
<thead>
<tr>
<th>Non-protein particles; detected</th>
<th>Protein particles; detected and characterized</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Image of non-protein particle" /></td>
<td><img src="image" alt="Image of protein particles" /></td>
</tr>
</tbody>
</table>

(0.1 µm) 0.75 µm – ca 70 µm

- Particles larger than 100 nm can be detected
- Detected particles can be counted
- The size and properties of particles can be determined
## Flow cytometry based sub-visible particle detection and analysis

<table>
<thead>
<tr>
<th>Non-protein particles; detected</th>
<th>Protein particles; detected and characterized</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Non-protein particle" /></td>
<td><img src="image2" alt="Protein particles" /></td>
</tr>
</tbody>
</table>

- **Particles larger than 100 nm can be detected**
- **Detected particles can be counted**
- **The size and properties of particles can be determined**

### Dyes and Particles

- **Protein specific dye**
- **Attribute specific dye**
- **Protein monomer**
- **Protein particles**
- **Non-protein particle**

### Particle Sizes

- (0.1 µm) 0.75 µm – ca 70 µm

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Flow cytometry based sub-visible particle detection and analysis

- Non-protein particles; detected
- Protein particles; detected and characterized

Particles larger than 100 nm can be detected
Detected particles can by counted
The size and properties of particles can be determined

(0.1 µm) 0.75 µm – ca 70 µm
Flow cytometry based sub-visible particle detection and analysis: establishing size ranges

0.1 – ca 200 µm depending on available equipment

Currently using:
0.75 µm – ca 70 µm
Detected sub-visible particles
Gating strategy

Detected sub-visible particles

Protein/non-protein

FSC

SSC

Protein specific signal

Protein

Non-protein

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Gating strategy

Detected sub-visible particles

Protein/non-protein

Gate on Protein

FSC

SSC

Protein specific signal

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Gating strategy

Detected sub-visible particles

Protein/non-protein

Gate on Protein

Property of protein particles

FSC

SSC

FSC

Attribute specific signal

FSC

Attribute specific signal
Flow cytometry based sub-visible particle detection and analysis: example

Analysis of protein particles, cross-beta sheet containing protein particles and non-protein sub-visible particles in one sample

All detected particles
Flow cytometry based sub-visible particle detection and analysis: example

Analysis of protein particles, cross-beta sheet containing protein particles and non-protein sub-visible particles in one sample

All detected particles

Number of detected particles in each size gate

<table>
<thead>
<tr>
<th>Name</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>10um - ≥10um</td>
<td></td>
</tr>
<tr>
<td>6um - 10um</td>
<td></td>
</tr>
<tr>
<td>4.5um - 6um</td>
<td></td>
</tr>
<tr>
<td>2um - 4.5um</td>
<td></td>
</tr>
<tr>
<td>1um - 2um</td>
<td></td>
</tr>
<tr>
<td>0.75um - 1um</td>
<td></td>
</tr>
</tbody>
</table>
Discrimination between protein and non-protein particles

Analysis of protein particles, cross-beta sheet containing protein particles and non-protein sub-visible particles in one sample

Detected sub-visible particles
Analysis of protein particles, cross-beta sheet containing protein particles and non-protein sub-visible particles in one sample
Analysis of protein particles, cross-beta sheet containing protein particles and non-protein sub-visible particles in one sample
Characterization of the protein particles

Analysis of protein particles, cross-beta sheet containing protein particles and non-protein sub-visible particles in one sample
Characterization of the protein particles

Analysis of protein particles, cross-beta sheet containing protein particles and non-protein sub-visible particles in one sample

Cross-beta-sheets containing particles

DCVJ

Property of protein particles

Gate on Protein

Protein specific signal

Protein/non-protein
Number and properties of sub-visible particles

Analysis of protein particles, cross-beta sheet containing protein particles and non-protein sub-visible particles in one sample

Non-protein particles
Protein and protein containing particles
Cross-beta-sheet containing protein particles
Use of flow cytometry based particle analysis for product development
Use of flow cytometry based particle analysis for product development

Particle concentration [#/ml]

- Non-protein particles
- Protein and protein containing particles
- Cross-beta-sheet containing protein particles

Non-Protein control
Protein control
Protein 1
Protein 2
Protein 3
Protein 4

Freshly reconstituted

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Use of flow cytometry based particle analysis for product development

<table>
<thead>
<tr>
<th>Protein Concentration [#/ml]</th>
<th>Non-Protein control</th>
<th>Protein control</th>
<th>Protein 1</th>
<th>Protein 2</th>
<th>Protein 3</th>
<th>Protein 4</th>
<th>Protein 1</th>
<th>Protein 2</th>
<th>Protein 3</th>
<th>Protein 4</th>
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</thead>
<tbody>
<tr>
<td>Freshly reconstituted</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild thermal stress</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Non-protein particles
- Protein and protein containing particles
- Cross-beta-sheet containing protein particles

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The properties of sub-visible particles may be different for all proteins.
Use of flow cytometry based particle analysis for product development

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**Protein 1**

<table>
<thead>
<tr>
<th>Particle concentration [#/ml]</th>
<th>0h</th>
<th>Mild stress</th>
<th>Harsh stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;0.750 µm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;1.0 µm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;2.0 µm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;4.5 µm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;6.0 µm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;10 µm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;10 µm -70 µm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;4.5 µm -70 µm</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>&gt;6.0 µm -70 µm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;10 µm -70 µm</td>
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</table>

**Protein 3**

<table>
<thead>
<tr>
<th>Particle concentration [#/ml]</th>
<th>0h</th>
<th>Mild stress</th>
<th>Harsh stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;0.750 µm</td>
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<tr>
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<td></td>
</tr>
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</tr>
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Legend:
- Non-protein particles
- Protein and protein containing particles
- Cross-beta-sheet containing protein particles

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Use of flow cytometry based participle analysis to study protein-silicon interaction

Does a native protein bind to silicon?
Does a native protein bind to silicon?

**Native protein**

**Silicon oil particles**
Use of flow cytometry based participle analysis to study protein-silicon interaction

Does a native protein bind to silicon?

Native protein

Silicon oil particles

Protein in a buffer with silicon
Protein binds to silicon sub-visible particles which become stained with BisANS.
Take home message

• Sub-visible particles can differ not only in size but also in their structural properties

• Flow cytometry based sub-visible particle analysis is a powerful tool for detection, quantification and characterization of proteinaceous particles

• The method provides orthogonal information to currently available techniques such as Micro-flow imaging and Nanoparticle tracking analysis
Thank you for your attention!