Analytical methods to characterize aggregates and subvisible particles

Andrea Hawe

9th P4EU Meeting

30th November 2015 * MPI * Martinsried
Background of

**Company**
- privately held, independent service provider
- established in 2008

**People**
- interdisciplinary team of highly qualified scientists
- ~ 50 FTE

**Science**
- expert scientific board:
  - Prof. Dr. G. Winter
  - Prof. Dr. W. Friess
  - Prof. Dr. W. Jiskoot
  - Prof. Dr. J. Carpenter
  - Prof. Dr. C. Schöneich

**Techniques**
- innovative analytical and technical equipment,
- focus aggregate and particle characterization

**Research**
- cutting edge research in the field of protein sciences with top publications

**Service**
- formulation development
- lyophilization development
- GMP analytics for biopharmaceuticals
Formulation is characterization

- Knowing the properties of a protein, is the first step towards stabilizing it

- Stability-indicating methods for the different types of degradation are the basis for successful protein product development

- Analytical tool box should cover all types of species, impurities and degradation products within the formulation
  - aggregates and particles
  - structural changes (secondary, tertiary structure)
  - chemical changes
  - charge variants
  - content, activity, potency
Subvisible particles ... why relevant?

- **Regulatory agencies** expect more than light obscuration data for particles > 10 µm and > 25 µm (LO) and visual inspection.
- Aggregates and particles are considered **cQAs**.
- **Emerging techniques** open new possibilities and insights into products and processes (e.g. trouble shooting).
- Nanometer-sized particles as early and **sensitive indicator for (in)stability**?

<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>Size range</th>
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<tbody>
<tr>
<td>Oligomers</td>
<td>10 to 100 nm</td>
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<tr>
<td>Sub-micrometer particles/ nanometer aggregates</td>
<td>0.1 – 1 µm</td>
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<td>Subvisible particles/ micrometer aggregates</td>
<td>1 – 100 µm</td>
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<td>Visible particles</td>
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Aggregate & particle properties to be analyzed

- molecular weight
- hydrodynamic size
- equivalent circular diameter (ECD)
- equivalent spherical diameter (ESD)
- ...

Important question that goes beyond the presented analytical methods:

What are biological consequences, e.g. activity, PK/PD, immunogenicity of sub-micrometer particles for the patient?
Method overview for aggregates and particles

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- HP-SEC
- AUC
- Dynamic light scattering (DLS)
- Laser diffraction, static light scattering (SLS)
- Field flow fractionation (AF4/HF5)
- Nanoparticle tracking analysis (NTA)
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- Flow cytometry
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- Flow imaging / Dynamic imaging analysis
- Visual inspection

- 1 nm
- 10 nm
- 100 nm
- 1 µm
- 10 µm
- 100 µm
- 1 mm
Challenge: (dis)continuity of size distribution

Filipe et al. (2013) TrAC, 49:118-125

Murine IgG sample stressed by agitation for 1 hour at 400 rpm
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Separation techniques: AF4 and HP-SEC
AF4: better recovery at high aggregate content

IgG from forced degradation study (thermal stress)

HP-SEC analysis

AF4 analysis

→ better characterization of submicron aggregates in AF4

→ UV signal can be used for quantification

Hollow fiber flow field flow fractionation (HF5)

Fig. 2. Flow schematic of the novel flow control module that can operate either in AF4 or in HF5 mode. The system consists of the following elements: (1) metering valve, (2) pressure sensor, (3) flow measuring device, (4) six port switching valve, (5) HF5 cartridge or AF4 channel, (6) pressure sensor, (7) flow measuring device, (8) metering valve, and (9) six port switching valve.
Examples comparing AF4 and HF5

BSA

mixture of protein standards

Johann et al. (2011) J Chromatogr A. 1218:4126-4131
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Coriolis Pharma
Biopharmaceutical Research and Development Service
**DLS and NTA**

**Dynamic light scattering (DLS)**

- Measurement of scattering signal of **whole sample** over time (autocorrelation function)

  **Derived results:**
  - diffusion coefficient, hydrodynamic size (averaged $R_H$), size distribution and PDI

**Nanoparticle tracking analysis (NTA)**

- Tracking of **individual particles** by imaging of light scattered from particles

  **Derived results:**
  - particle concentration, hydrodynamic size & particle size distribution

\[
(x, y)^2 = \frac{2m K_b T}{3F_v \eta}
\]
Benefits of NTA

- Sample visualization and quantitative information (size range: ~ 50 – 1000 nm)
- Complementary method to DLS → better size resolution because of individual particle tracking
- Especially suitable for VLPs, viruses, liposomes, drug delivery systems
- Fluorescence option to detect labeled compounds in complex fluids
- Combination with zetapotential measurement possible

c) 200 and 400 nm beads

Number ratio: 2:1

Challenges for NTA analysis

- Optical system, impacted by refractive index difference and light scattering by monomeric protein, and large particles

- Analyzed volume is low → representative sampling critical and very high extrapolation factor for concentration determination (Options: syringe pump and longer acquisition times)

- Restricted concentration ranges (10^6-10^8/10^9 particles per ml) → dilution may be required

- Operator-dependency (improved in new systems)

- Limited possibilities for differentiation of heterogeneous samples (e.g. protein + silicone oil)

10 mg/ml IgG, simple buffer

large (micrometer) particles
Submicron aggregates analyzed by DLS and NTA

Example:
1 mg/ml IgG1 phosphate buffer, pH 7.0

Zave: 10.1 nm
PDI: 0.06

Zave: 66 nm
PDI: 0.19

Mean: 130 nm
1.75 x 10^8 particles/ml
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Resonant mass measurements (RMM)
Suspended microchannel resonator (SMR)

- Individual particles are “weighed” in a mechanically resonating microfluidic channel
  → frequency changes depending on particle’s buoyant mass
- **Measured parameter**: frequency shift which correlates to buoyant mass of individual particles
- **Derived parameters**: particle size (based on a known or assumed particle density), particle size distribution, particle classification (positively or negatively buoyant particles)
Benefits of RMM

• New measurement principle (light-independent)  
  → real orthogonal method to DLS, NTA (nm-size range) & light obscuration, flow imaging microscopy (µm-size range)
• Quantification of sub-micrometer particles ~ 300 nm to 2-4 µm
• Differentiation positively buoyant from negatively buoyant particles (e.g. silicone oil and protein → > 500 nm)
• Density determination

Challenges for RMM

- Low sampling efficiency (nanoliters) and high extrapolation factors → long measurement times required for representative sampling, especially for highly pure samples
- Robustness of the measurements: capillaries vulnerable to blockage
- No differentiation of different categories of positively and negatively buoyant particles (only one density for each class can be used)
- Density of the measured particles needs to be known to estimate size

**Figure 7.** Impact of protein particle densities on size bins for a proteinaceous sample (mAb1-heat). The impact of protein particles densities of 1.28 g/cm³ (black), 1.32 g/cm³ (red), and 1.35 g/cm³ (blue) was investigated. Horizontal box plots show 25/75% (box), mean values (squares), median (vertical line), and min/max (whisker).
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### Techniques

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Methods for micrometer particle analysis

Light obscuration

Flow imaging microscopy
Dynamic imaging analysis

Electric zone sensing

2D light blockage analysis

Flow cell

2D image analysis

3D conductivity analysis

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Method comparison standards

- typically good agreement of the methods for standards (e.g. PS), but not for protein samples
Method comparison for „real life“ samples

Example: comparison of an unstressed and stressed protein formulation

- 5 mg/ml protein
- Formulation is stressed by freeze-thawing → very transparent particles
RI as main challenge for light based techniques

- Significant underestimation of particle concentration in pharmaceutically relevant formulations when $RI_{\text{formulation}} > 1.35$
  
  e.g. 10% sugar, 100 mg/ml protein or 5% sugar + 50 mg/ml protein

# Flow imaging microscopy – image properties

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<tr>
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Zölls / Weinbuch et al. (2013) *AAPS Journal*, 15:1200-1211
Flow imaging microscopy – sizing properties

A) MFI 4100
B) MFI 5200
C) FlowCAM VS1
D) FlowCAM PV

Zölls / Weinbuch et al. (2013) AAPS Journal, 15:1200-1211
Example for high sensitivity of particle methods

- 100 kDa protein formulated at 1.2 mg/ml
- Comparison of different lyophilized formulations stored at 40°C/75% r.h.

**HP-SEC**

- HP-SEC monomer [%] vs. storage time [months]
  - Total peak area unchanged

**light obscuration**

- Particles larger 1 um per ml vs. storage time [months]
Particle identification by Raman microscopy

- Morphologi G3-ID: combines microscopy and Raman spectroscopy

- Application: chemical identification of particles (in solution) based on Raman spectroscopy (qualitative analysis) → trouble shooting

- Vision: apply dynamic imaging analysis as high-throughput method → add information generated from Raman microscopy to confirm identity

thin path wet cell (100-300 µl)
Example silicone oil

Selection of three particles for chemical identification
Example silicone oil

Raman spectra (background subtracted) overlay of selected particles with silicone oil as reference
Identification of other particles in solution

- 1004 cm⁻¹: Phe
- 1450 cm⁻¹: CH₂ backbone
- 1650 cm⁻¹: Amide I
- 490 cm⁻¹
- 712 cm⁻¹
- 1100 cm⁻¹
- 490 cm⁻¹
- 712 cm⁻¹

Particles:
- Particle 1270
- Particle 3083
- Particle 3340
- Particle 1871
- Particle 2617
Conclusions

- Several methods are available for detection, counting/quantification, sizing & partially classification/identification of aggregates/subvisible particles
- **Robustness** of the methods for sub-micrometer particles remains challenging
- Method(s) need to be selected case-by-case depending on the purpose of analysis and type of protein/formulation
- We all are encouraged to share experience and publish results to generate a better understanding in the community
Acknowledgements

• Ariadna Martos (Coriolis Pharma)
• Daniel Weinbuch (Coriolis Pharma)
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• **Coriolis Pharma Team**

**Coriolis’ scientific Advisory Board**

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