Characterization and Biological Relevance of Protein aggregates and other particles 100-200,000 nm in size (sub micron and subvisible)

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(and AAPS Protein aggregate and biological consequences community)

Outline

- Introduction/historical perspective
- Description of Techniques in USP
- Results of industry survey
- Summary and future plans

Biotherapeutics should be

- Efficacious
  - Achieve desired result at reasonable dose
  - With long enough half life (PK) to be effective
- Safe:
  - No unexpected side effects
  - No non-specific binding
  - No Toxicity
  - Minimized Immunogenicity (both neutralizing and non-neutralizing Abs)
- Manufacturable:
  - Stable shelf life for up to 2 years
  - Able to make consistently and efficiently
  - Fit existing facilities and process platforms much as possible

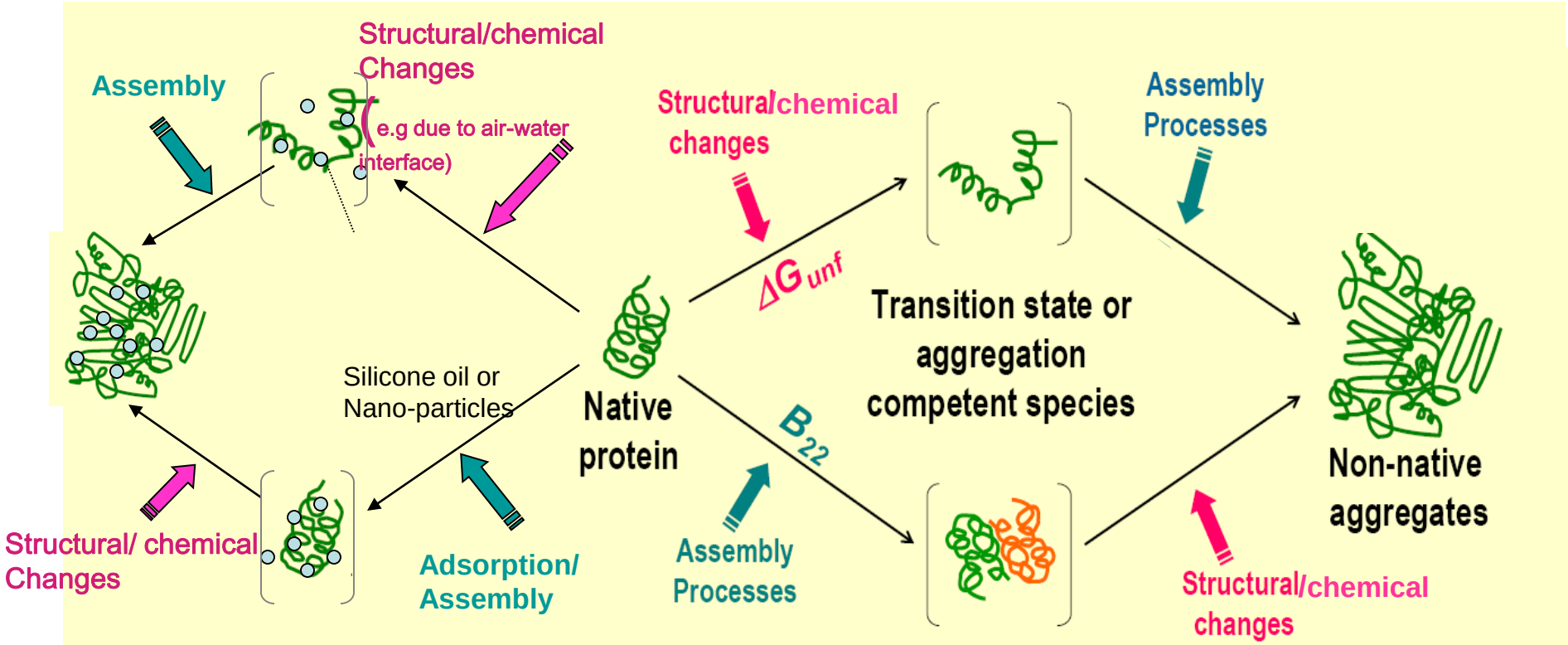
Particles are indicative or process control, sterililty, etc

Protein aggregates could impact safety and efficacy

Proteins aggregate via different pathways

Heterogeneous nucleation or interface dependent aggregation

Conformational or colloidal stability dependant aggregation



Particles can have different morphology based on the mechanism of formation

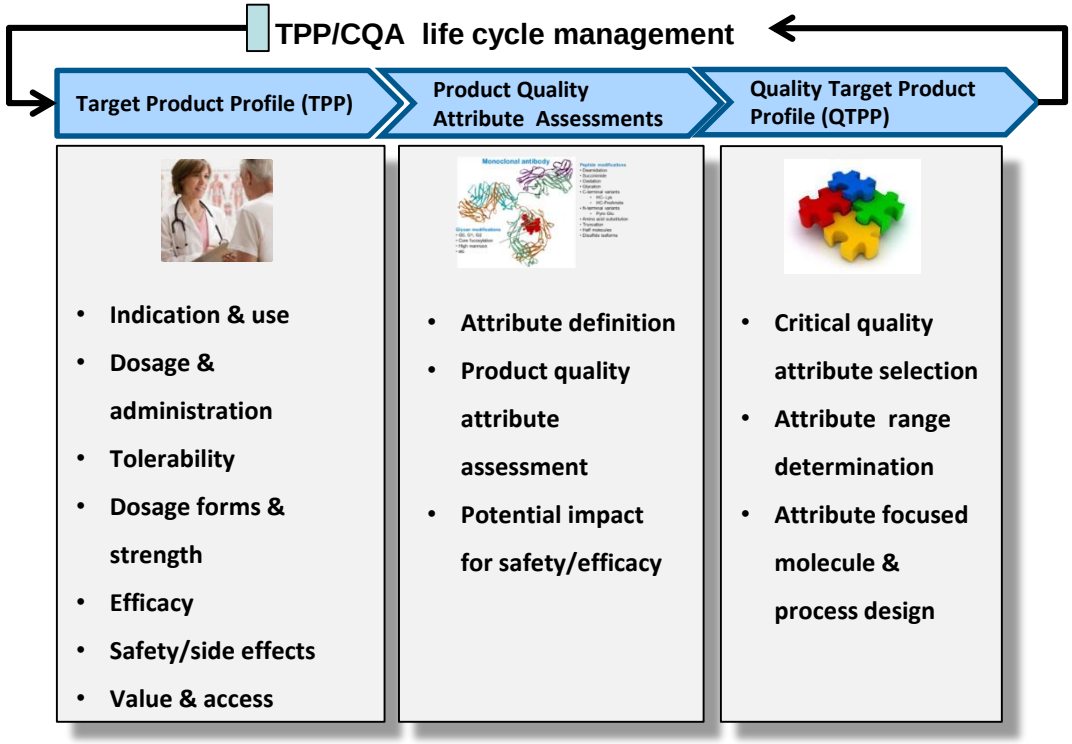
Synthesis of work from multiple scientists including R. Thirumangalathu, J. Bee, S Krishnan, EY Ch, H-C Mahler, M. Joubert , Q Li, S. Shire, M Cromwell, L. Narhi, et al

Aggregates are a very heterogeneous population requiring multiple descriptors*

- Size (Quaternary structure)
  - <100 nm (Nanometer)
  - 100-1000 nm (Sub-μm)
  - 1-100 μm (micron, SbVP)
  - >100 μm (Visible particles have company-specific size range)
- Reversibility
  - Reversible should be restricted to aggregates for which an equilibrium constant can be measured. That is, the disassociation of proteins may be observed on the experimental time scale simply by reverting to original conditions.
  - Irreversible
  - Dissociable under physiological conditions
  - Dissociable with denaturant when conditions that disrupt structure are required to dissociate the aggregate
- Secondary/Tertiary structure
  - native
  - partially unfolded
  - unfolded
  - amyloid
  - Inherently disorded
- Covalent Modification
  - Chemical modification
  - Cross-linked
  - Reducible crosslink
  - Non-reducible crosslink
  - Intra-molecular modification
  - No modification
- Morphology
  - Aspect ratio
  - Surface roughness
  - Internal morphology
  - Homo and heteroaggregates
  - Translucent
  - Heterogeneous
- Optical properties: similar for all protein particles

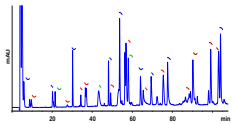
*Narhi, Linda O., Schmit, J., Bechtold-Peters, K., Sharma, D., Classification of Protein Aggregates (2012) J. Pharm. Sci. 101, 493-498.

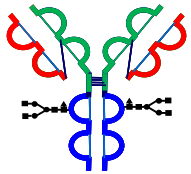
Aggregates are a Critical Quality attribute and should be treated as such



Characterization of particles and "what is normal" increase with progression through development, enabling a phase appropriate, risked based approach

Assay requirements change during the product lifecycle.



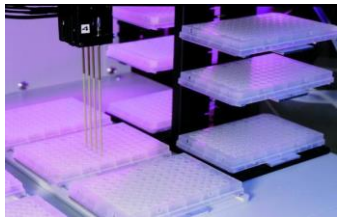


release

- *Qualified*
- *Compliant*
- *Highly Reliable*
- *Also used for stability testing*

characterization

- *More Detailed Structural Information*
- *Often Complex, Slow assays*
- *Used Less Frequently*
- *Data supports regulatory filings*



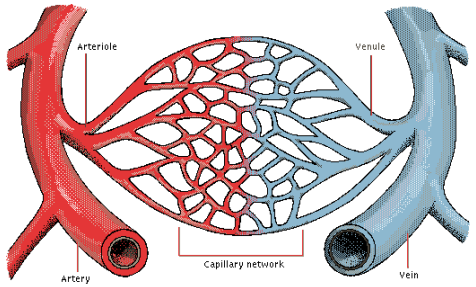
screening

- *High Throughput*
- *Predictive*
- *Minimal Qualification*
- *Used for clone screening, support of process development*

Aggregate/Particles should be treated like all CQAs, acquiring knowledge early on to inform what is normal at lot release

History of Subvisible particles in USP/EP/JP

- Harmonized EP 2.9.19 Particulate Contamination: Sub-visible Particles and USP <788> Particulate Matter in injections both contained guidance on acceptability of ≥10 and ≥25 micron particles (6000 and 600 per container)
- Essentially created to control levels of foreign particles in small molecule parenteral (extrinsic and intrinsic particles)
- Safety concerns were around capillary occlusion by these rigid SbVP,
- Also seen as indicating contamination, loss of process control, etc.
- No other regulatory guidance existed for subvisible particles apart from the pharmacopoeias
- Lot release method, robust and reliable



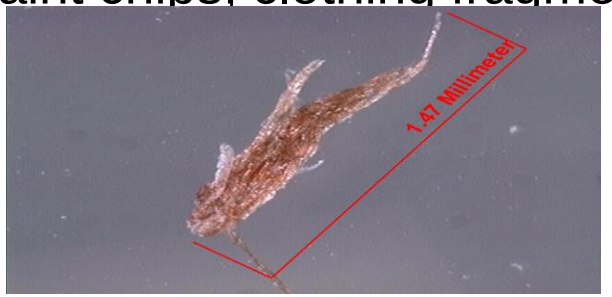
For biologics, the focus on SbVP has changed to potential immunogenicity

COMMENTARY (by Authors from Academia and the FDA) Overlooking Subvisible Particles in Therapeutic Protein Products: Gaps That May Compromise Product Quality, John F. Carpenter, Theodore W. Randolph, Wim Jiskoot, Daan J.A. Crommelin, C. Russell Middaugh, Gerhard Winter, Ying-xin Fan, Susan Kirshner, Daniela Verthelyi, Steven Kozlowski, Kathleen A. Clouse, Patrick G. Swann, Amy Rosenberg, Barry Cherney **J Pharm Sci. 2009 Apr;98(4):1201-5. doi: 10.1002/jps.21530.**

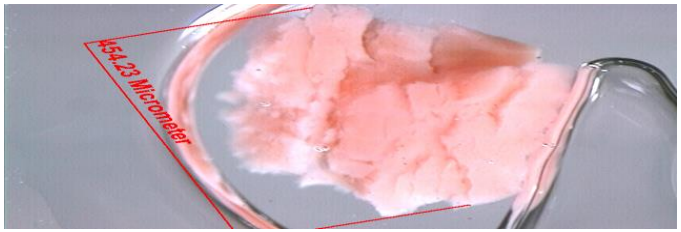
- Original USP particulate testing was not designed to measure protein particle size distribution, or to address the potential risk of large protein aggregates to impact protein immunogenicity.
- All formulated antibody drug products contain low levels of aggregates.
- The clinical immunogenic risk of aggregates is uncertain, resulting in a high risk factor being assigned to the presence of protein aggregates in biologics.
- To reduce this uncertainty, the following should be defined:
  - Aggregate attributes that cause a response
  - Amount of aggregate required to break the threshold of activation
  - Extent and nature of the response
  - Extensive studies with different proteins, stresses, and model systems suggest the response depends on protein sequences, aggregate characteristics (including size, modification, and morphology), administration, and model systems or patient attributes. (Jiskoot et at, 2016, Ehab et al, 2016, etc)
- Analytical methods that can assess particulate characteristics (including composition, amount and reversibility of the protein aggregate) are critical for developing scientifically sound approaches for evaluating and mitigating risk to product quality caused by large protein aggregates and other particles

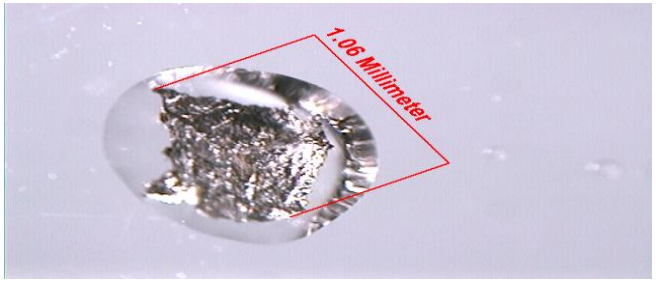
USP definitions: Visible and SbVP Particles can be assigned to one of three categories <1787>, <740>

- **Extrinsic particles** (from the outside) are materials that are not part of the drug product, package, or process, but are present due to contamination. These are truly foreign particles that are unexpected in drug product (e.g., insect parts, paint chips, clothing fragments, hair).



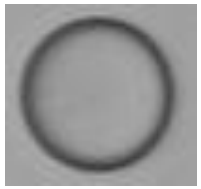
- **Intrinsic particles** (from the inside) are undesirable, non-protein material from degradation of formulation components, or related to the manufacturing and packaging processes and the device itself (e.g., glass lamellae, particles arising from packaging materials for drug product components, rubber from stoppers, silicone oil).



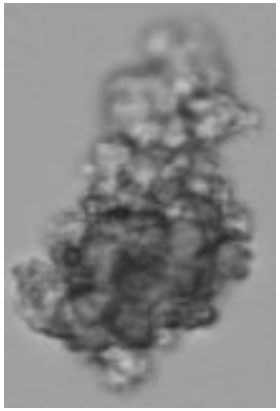


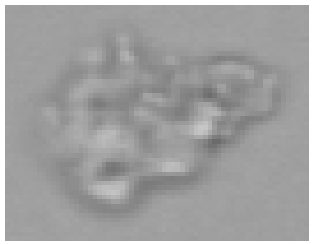
USP SbVP definitions

- **Silicone oil droplets** are important intrinsic particles resulting from the silicone oil that is a necessary lubricant in glass pre-filled syringes. They can confound the analysis of the total subvisible particle population, and also have the potential to interact with the protein depending on formulation conditions[1-4]



- **Inherent particles** are particles which originate from the drug product, either the protein therapeutic itself or formulation components. These particles can be an expected characteristic of the drug product.





The primary lot release method in the Pharmacopeia is Light Obscuration Method

From Beckman Coulter
http://www.beckman.com/particle/instruments/lab-liquid-particle-counters/hiac-9703

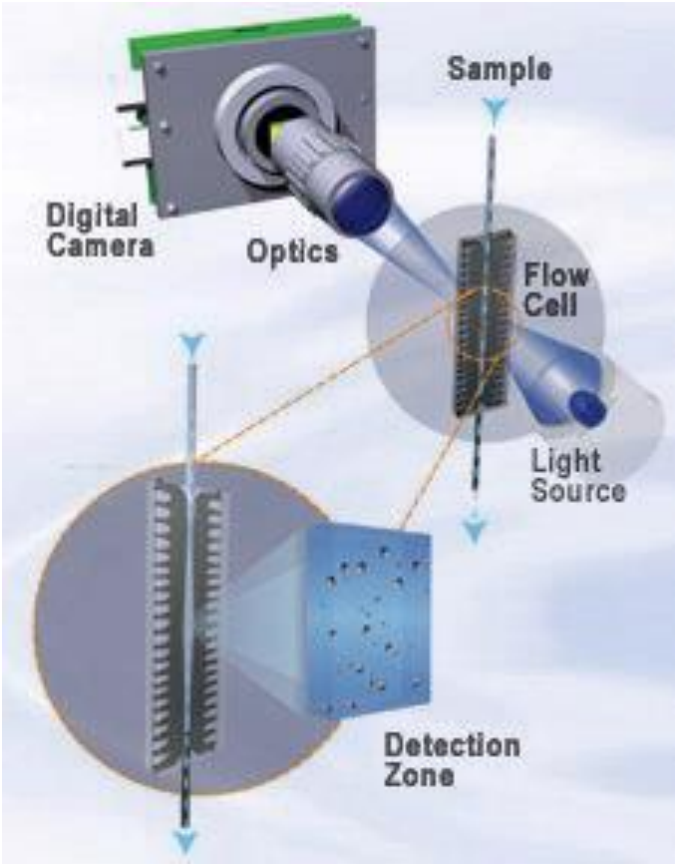
- **<787> describe a method better suited for biologics:**
  - Test individual units (as much as possible)
  - Reduced sample volume 5mL
  - For many biologics individual units are less than 1 ml
    - Release and stability testing: (≤) 5 mL/test
      - Characterization & investigation testing: ≤ 5 mL/test
    - Qualification and validation: < 100 ml
  - Extend to multiple (e.g. 7) size channels: ≥ 2, 5, 10, 15, 20, 25, 50 μm
  - Modify & improve sample handling procedure to reduce false negatives and positives (micro bubbles, etc)
  - Improve performance compared to <788>
- **Intended use for drug products**:
  - Release and stability testing
  - Process and product characterization
  - Investigations

  Could be applied to all parenterals

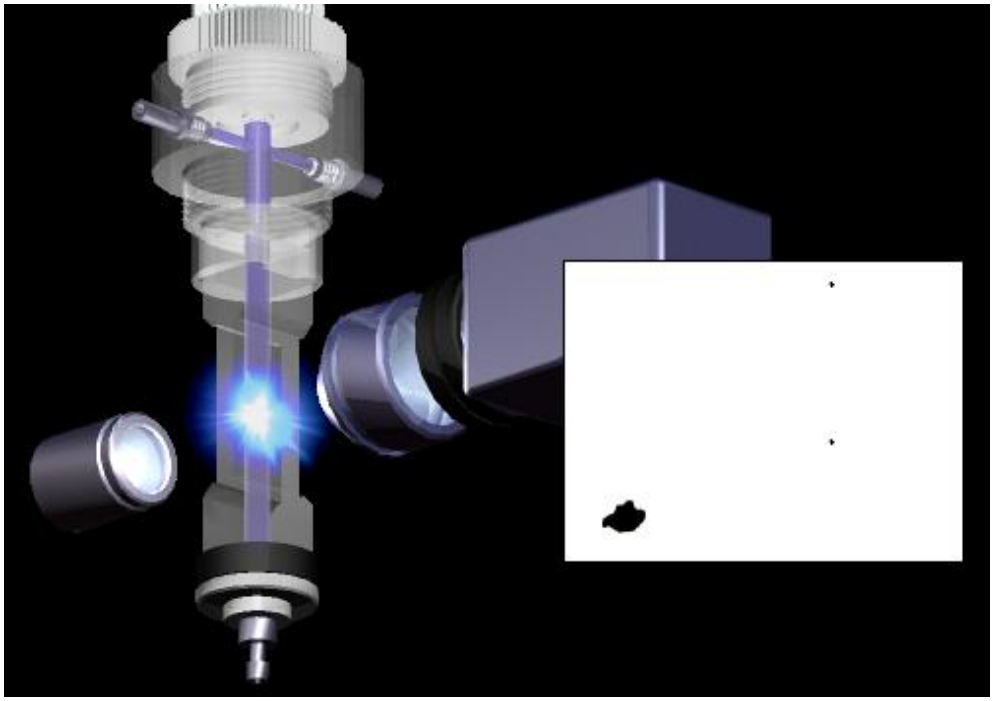
<1787> describes methods for particle/aggregate analysis beyond lot release
<1788> includes best practices for Dynamic imaging and membrane microscopy

The most commonly used orthogonal method is dynamic flow imaging, Camera Based Technologies

MFI (micro-flow imaging)



Flowcam



Taken from instrument manufactures' information

Techniques for particle size and distribution analysis from <1787>

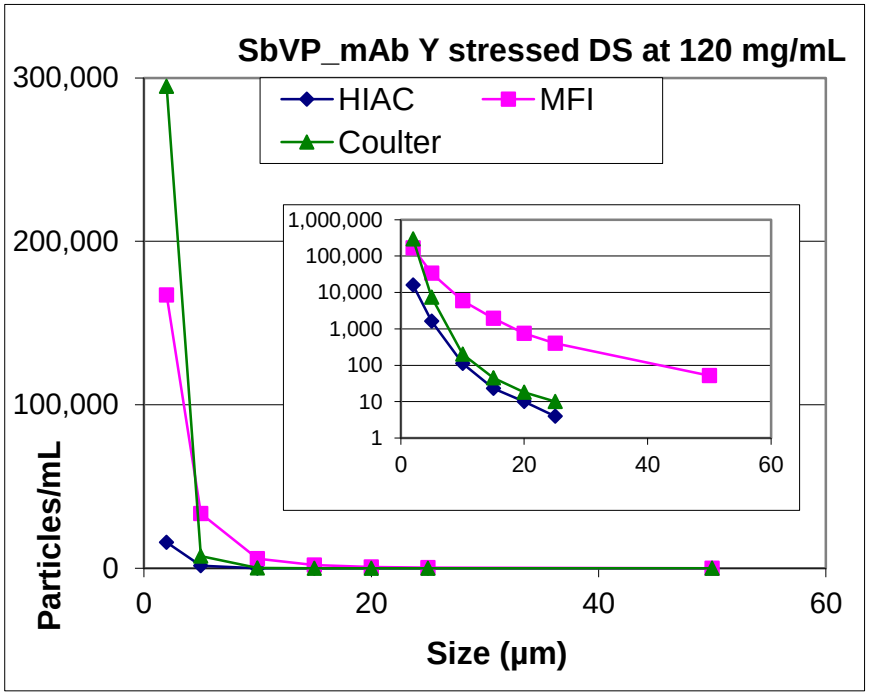
Technique	Principle of Operation	Range
Turbidimetry and Nephelometry	Estimation of the particle size distribution is attained by measuring the interaction of light with suspended particles, by the loss in intensity of transmitted light (turbidimetry) or light scattering (nephelometry).	0.035µm to 50µm
Light Obscuration	The size of the particle in the product fluid is determined by the amount of light that it blocks when passing between the source and the detector.	1 to 300µm
Coulter: electrical sensing zone	The size of the particle in product fluid or selected electrolyte is determined by the change in resistance as the particle passes through a micro-channel (orifice).	1 to 1600µm
Mastersizer (laser diffraction)	Intensity and angle of scattering generates a particle size distribution curve	0.01-3000 micrometers

Techniques for size and morphology analysis from <1787>

Technique	Principle of Operation	Range
Light Microscopy	Photon imaging of substances directly in product fluids or mounts or of isolated specimens on substrates.	0.3µm to mm's
Dynamic Imaging Analysis: Flow Microscopy	Digital image capture of the particles' magnified image in streaming product fluid, revealing size, shape, optical properties.	0.7 to 100µm for size distribution 4 to 100µm for morphology
Electron Microscopy (EM): Scanning EM, Scanning transmission EM and transmission EM	Electron imaging of specimen isolates on substrates. High vacuum or near-ambient pressures required.	Angstroms to mm's
Flow Cytometry: Forward scattering channel	Passage of particle across light beam increases light scattering in forward direction. Low refractive index difference, irregular morphology	1-100 micrometers

The absolute numbers and size of micron aggregate depends on the instrument used

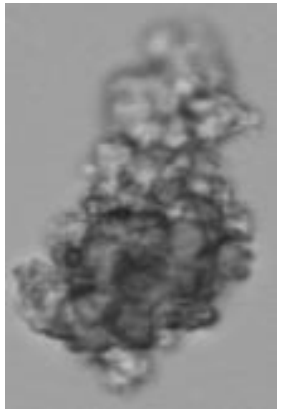
HIAC, MFI and Coulter Counter gave different particle concentrations and particle size distributions for protein particles in mAb Y drug substance at 120 mg/mL



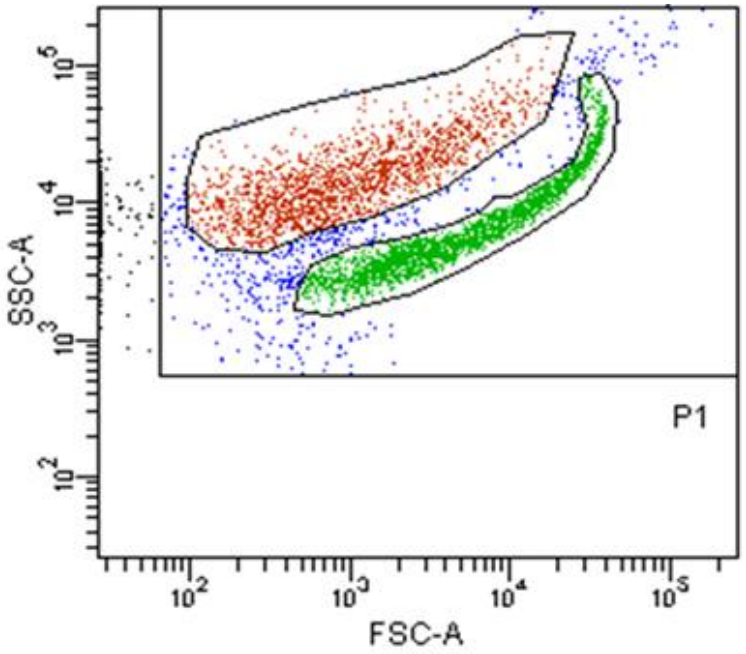
- HIAC has the lowest counts of particles through all sizes and dilutions
- Coulter has highest counts of smaller particles (2-5 µm)
- MFI has the highest counts of larger particles (≥ 5 µm).
- Dean Ripple has published on underlying causes for these types of discrepancies, and ways to address them

Relative ranking of samples is usually consistent across techniques

Output from size and morphology analysis



Dynamic flow image



Flow cytometry output

Techniques for characterization from <1787>

Technique	Principle of Operation	Range
FTIR Microspectroscopy	Photon imaging of isolated specimens on substrates	10μm to mm's
Dispersive-Raman Microspectroscopy	Photon imaging of isolated specimens on substrates, or in product fluids or fluid mounts	0.5μm to mm's
Electron Microscopy with Energy-Dispersive X-ray Spectrometry [EDS]	X-ray Photon emission from specimens energized by a focused electron beam	Angstroms to mm's for imaging, 1μm to mm's for elemental composition
Electron Microscopy with Electron Energy Loss Spectroscopy [EELS]	Inelastic scattering from specimens energized by a focused e-beam; e-loss is characteristic of the source element. Complementary to EDS.	Angstroms to mm's for imaging, 0.5μm to mm's for elemental composition

What techniques are suitable for what phase of development?

- AAPS PABC focus group did a survey with responses obtained from 7 industrial and contract analytical labs
- The participating labs rated the application of each analytical particle/aggregation method at each phase of the product life cycle using a scale of 1-5 (1 being not recommended, 5 being most often used or a requirement).
- The ratings were consolidated and discussed, including in a webinar, resulting in a recommendation for the phase-appropriate application of particle analytical methods

Analytical methods and phases of development included in survey

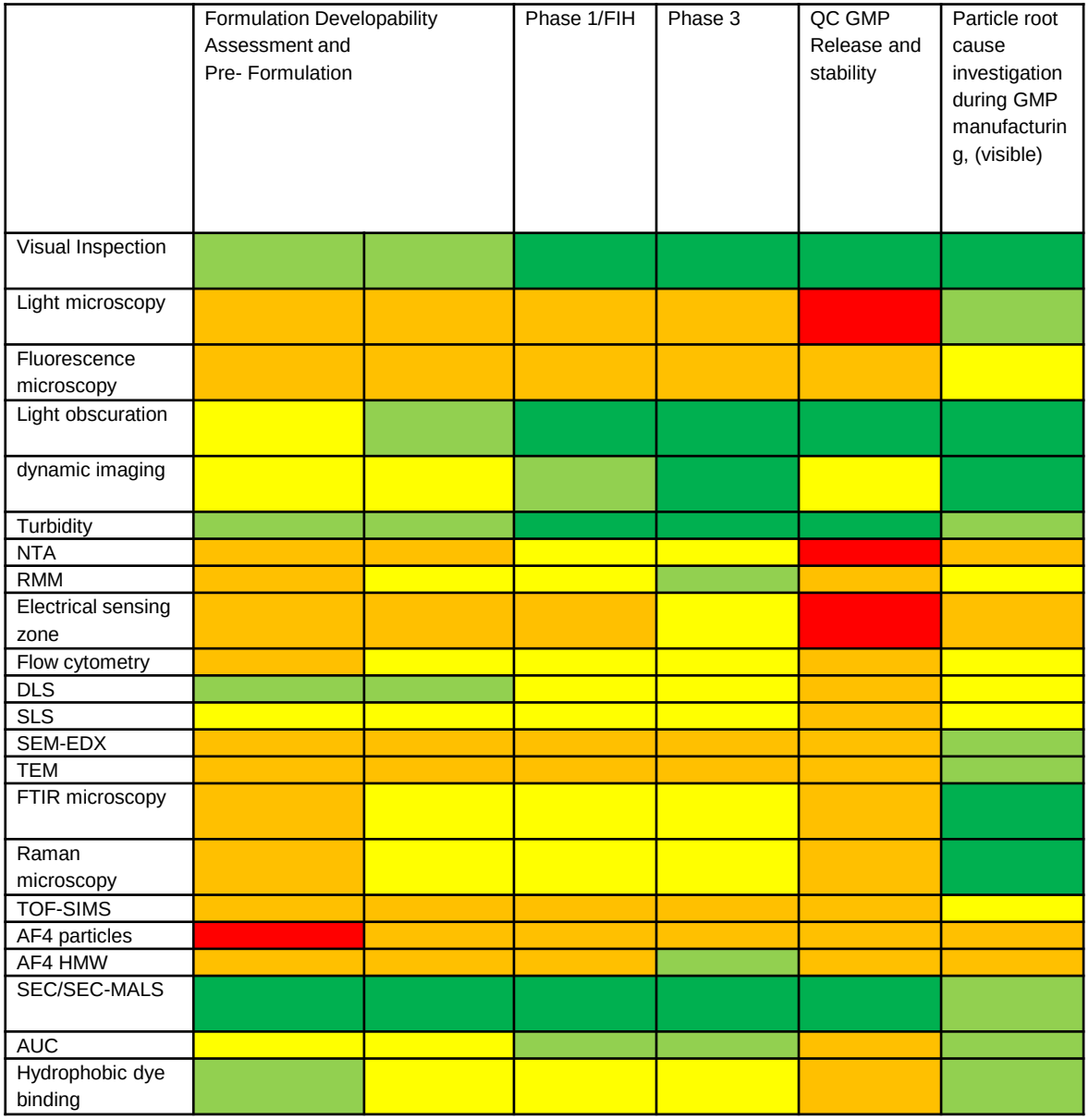
Dark green = most often used or a requirement (average scale rating 5)

Light green = typically used (average scale rating 4)

Orange = occasionally used (average scale rating 3)

Yellow = rarely used (average scale rating 2)

Red = not recommended (average scale rating 1)



There were clear industry trends in what methods are used at what phase of development

- Initially predictive methods, that can be automated, are used to select the candidate and process to minimize aggregation, and provide relative rankings, require small volume, lot release methods are usually not appropriate (large volume) at this point
- During process and product development, from FIH to phase 3 multiple orthogonal methods are used to understand the aggregate, and to implement control strategy , including compendia methods
- For lot release LO is sufficient if backed up by previous characterization, with other tools ready in case of NC or investigation.

Submicron

- Through IQ consortium determination of submicron particles present in marketed product using RMM and NTA has been completed, manuscript submitted,
- begin understanding clinical exposure and baseline of these species in material administered to humans
- To see if there are correlations between DP characteristics like volume, concentration, liquid or lyo and submicron population
- Established best practices for sample handling and use of instruments
- Assessed variability of techniques

How do we test to see if particles do have risk of immunogenicity?

- In silico modeling based on sequences for Tcell recognition
- In vitro cell based model systems (both Hu PBMC and cell lines)
- In vitro organelle model systems
- In vivo mouse models
  - Wild type, Xeno, and Xeno/Het Mice

All provide relative ranking for potential immunogenicity.

POINTS TO CONSIDER AND FUTURE PLANS

Key points to consider

- There is increased scrutiny on SbVP in protein products due to potential risk of immunogenicity
- Characterization with orthogonal methods is important (Coulter counter, MFI and other flow microscopy techniques, etc. in addition to light obscuration/HIAC)
- For all techniques it is important to verify results with expert analysts
- Characterization during development can both minimize particles present, and also result in understanding of “what is normal” and control strategy
- This should enable use of LO as the lot release method based on deep understanding of product gained during characterization

Points to consider, cont'd

- The field is moving to a common nomenclature for protein aggregates,
- Sample handling is critical, including effect of dilution on particle size distribution, micro-particle removal, etc.
- Particle standards that are similar in optical properties and density to protein aggregates have been developed by NIST

Summary and future plans

- Protein aggregates occur due to multiple factors, inherent molecular properties, process conditions, and interactions with formulation and device.
- Our analytical ability and understanding of the biological consequences of micron protein aggregates has improved significantly over the last few years.
  - Exploration of the applications of these techniques during product development continues
- All techniques have strengths and weaknesses. High concentration analysis is particularly difficult for all of them.
- USP expert committee finalized <787> (Biologics specific chapter), <1787>, informational chapter, and stimulus articles on submicron particles, and
  - is currently working on adding flow imaging (without specifications) to <1788>
- Bridging studies demonstrate that products that pass <787> will pass <788> as well, so companies do not have to file with both
- **Discussion to add adjustments in <787> to harmonized chapters is ongoing**

Future plans

- AAPS focus groups on Protein aggregation and Biological Consequences is planning cross lab experiment (16 labs from industry, academia, regulatory agencies and NIST), using aggregate from same proteins (6), generated by same stresses, and characterize in the same assays
    - examine variability of characterization assays, in vitro and in vivo models, understand the variability of assays
    - Identify CQA of aggregates that have some activity in in vitro and in vivo assays
    - The outcome will be 3 publications, one for each phase of the study.

Some related publications

- Rigidly organized protein arrays in the micron range may be highly immunogenic
  - VSV-G and VLV and regularly spaced acrylamide polymers (5-10 nM) are immunogenic

    Bachmann et al., Annu. Rev. Immunol, 15 (1997) 235-70.
    Denis et al., Virology, 363 (2007) 59-68.
    Dintzis et al., PNAS, 73 (1976) 3671-5.
    Chackerian et al, J Immunol, 169 (2002) 6120-6.

  - Immune response of protein coated nanobeads and preferential internalization of protein coated aluminum adjuvants by DCs

    Fifis et al., J Immunol, 173 (2004) 3148-54.
    Morefield et al., Vaccine, 23 (2005) 1588-95.

- Reports of protein aggregate immunogenicity *in vivo* give conflicting results
  - Aggregates of IFN-γ: metal-catalyzed and pH/50°C induced aggregates (but not untreated, crosslinked, hydrogen peroxide or boiled) can break tolerance in transgenic mice.

    Hermeling et al., Pharm Res, 22 (2005) 1997-2006.
    Hermeling et al., J Pharm Sci, 95 (2006) 1084-96.

  - Aggregates of FVIII: heat induced aggregates were less immunogenic than the monomeric protein.

    Purohit et al., J Pharm Sci, 95 (2006) 358-71.

  - Aggregates of GH: freeze-thaw and agitation induced aggregates were not able to break the tolerance of transgenic mice (freeze-thaw and GH absorbed onto glass or alum particles showed an enhanced response in wild-type mice).

    Fradkin et al., J Pharm Sci, 98 (2009) 3247-64.
    Fradkin et al., J Pharm Sci, (2011)

  - Only highly chemically modified aggregates (oligomers) broke tolerance in transgenic mouse model

    Bessa et al Pharm Res (2015) DOI 10.1007/s11095-015-1627-0

  - A weak transient response was obtained with aggregates in the 2-10 micron size range with some native structure and chemical oxidation in a Xeno-het model

    BI et al J Pharm Sci 2013 102 (10): 3545-55

Related Publications, cont'd

- **John F. Carpenter, Theodore W. Randolph, Wim Jiskoot, Daan J.A. Crommelin, C. Russell Middaugh, Gerhard Winter, Ying-xin Fan, Susan Kirshner, Daniela Verthelyi, Steven Kozlowski, Kathleen A. Clouse, Patrick G. Swann, Amy Rosenberg, Barry Cherney.** Overlooking Subvisible Particles in Therapeutic Protein Products: Gaps That May Compromise Product Quality **J Pharm Sci. 2009 Apr;98(4):1201-5. doi: 10.1002/jps.21530.**
- **Singh, Satish K. ; Afonina, Nataliya; Awwad, Michel; Bechtold-Peters, Karoline; Blue, Jeffrey T.; Chou, Danny; Cromwell, Mary ; Krause, Hans-Juergen ; Mahler, Hanns-Christian; Meyer, Brian K.; Narhi, Linda; Nesta, Doug P.; Spitznagel, Thomas . An Industry Perspective on the Monitoring of Subvisible Particles as a Quality Attribute for Protein Therapeutics (2010) J. Pharm. Sci. 3302-3321**

- **Scott Aldrich, Shawn Cao, Andrea Hawe, Desmond Hunt, Linda Narhi, Dean Ripple, Satish K. Singh. Analytical Gaps and Challenges for Particles in the Submicrometer Size Domain. Pharmacopeial Forum 2016;42(6)**
- **Jiskoot W, Kijanka G, Randolph TW, Carpenter JF, Koulov AAV, Mahler HC, Joubert MK, Jawa V, Narhi, LO Mouse Models for Assessing Protein Immunogenicity: Lessons and Challenges (2016) J Pharm Sci 105 1567-1575**
- **Moussa EM, Panchal JP, Balakrishnan SM, Blum JS, Joubert MK, Narhi LO, Topp EM. "Immunogenicity of therapeutic protein aggregates" J Pharm Sci 2016 (DOI: 10.1016/j.xphs.2015.11.002).**
- **Narhi LO, Corvari V, Ripple DC, Afonina N, Cecchini I, Defelippis MR, Garidel P, Herre A, Koulov AV, Lubiniecki T, Mahler H-C, Mangiagalli P, Nesta D, Perez-ramirez B, Polozova A, Rossi M, Schmidt R, Simler R, Singh S,Spitznagel TM, Weiskopf A, Wuchner K Subvisible (2–100 m) Particle Analysis During Biotherapeutic Drug Product Development: Part 1, Considerations and Strategy (2015) J Pharm Sci** *DOI 10.1002/jps.24437*

- **Joubert MK, Deshpande M, Yang J, Reynolds H, Bryson C, Fogg M, Baker MP, Herskovitz J, Goletz TJ, Zhou L, Moxness M, Flynn GC, Narhi LO, Jawa V. "Use of In Vitro Assays to Assess Immunogenicity Risk of Antibody-Based Biotherapeutics" 2016 PLOS One**
- **V Bi, V Jawa, MK Joubert, A Kaliyaperumal, C Eakin, K Richmond, O Pan, J Sun, M Hokom, TJ Goletz, J Wypych, L Zhou, BA Kerwin, LO Narhi and T Arora "Development of a human antibody tolerant mouse model to assess the immunogenicity risk due to aggregated biotherapeutics"** ***J Pharm Sci*** **2013 102 (10): 3545-55.**
- **MK Joubert, M Hokom, C Eakin, L Zhou, M Deshpande, MP Baker, TJ Goletz, BA Kerwin, N Chirmule, LO Narhi and V Jawa "Highly aggregated antibody therapeutics can enhance the in vitro innate and late-stage T-cell immune responses"** ***J Biol Chem*****. 2012 287 (30): 25266-79.**
- **Q Luo, MK Joubert, R Stevenson, RR Ketchem, LO Narhi and J Wypych "Chemical modifications in therapeutic protein aggregates generated under different stress conditions"** ***J Biol Chem*** **2011 286 (28): 25134-44.**
- **MK Joubert, Q Luo, Y Nashed-Samuel, J Wypych and LO Narhi "Classification and characterization of therapeutic antibody aggregates"** ***J Biol Chem.*** **2011 286 (28): 25118-33.**
- **Narhi et al., JPharmSci, 101, 493, 2012**
- **Narhi et al., JPharmSci, 10.1002/jps.24437, 2015**

- **Ripple, D.C. & Hu, Z. "Correcting the Relative Bias of Light Obscuration and Flow Imaging Particle Counters," Pharm Res (2016) 33: 653. https://doi.org/10.1007/s11095-015-1817-9**
- **Ripple DC, Montgomery CB, Hu Z, "An interlaboratory comparison of sizing and counting of subvisible particles mimicking protein aggregates," J Pharm Sci. (2015) 104(2):666-77. https://doi.org/10.1002/jps.24287**
- **Ripple, D.C. & Wayment, J.R. & Carrier, M.J.. (2011). Standards for the optical detection of protein particles. American Pharmaceutical Review. 14. 90-96. Available at:**

https://www.americanpharmaceuticalreview.com/Featured-Articles/36988-Standards-for-the-Optical-Detection-of-Protein-Particles/

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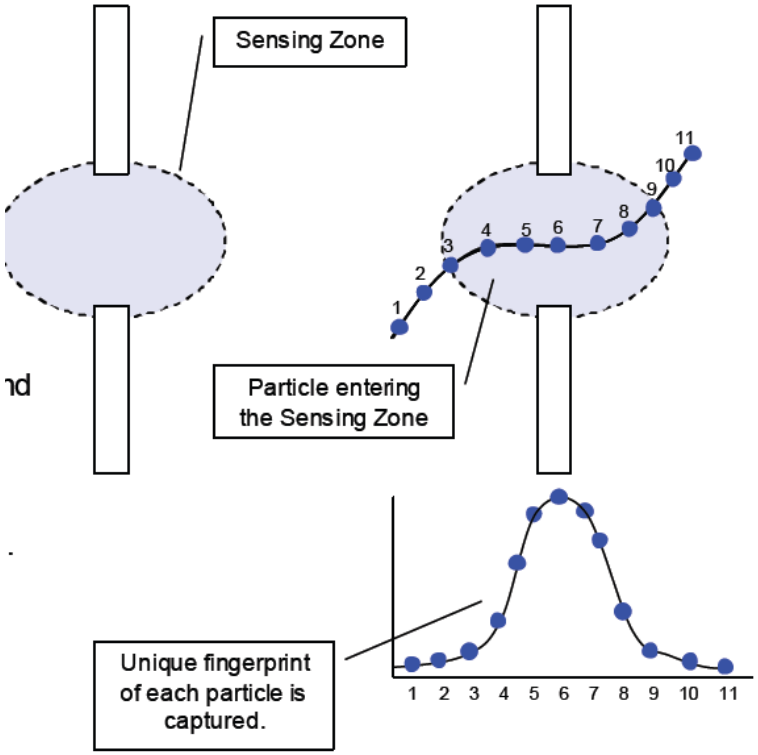
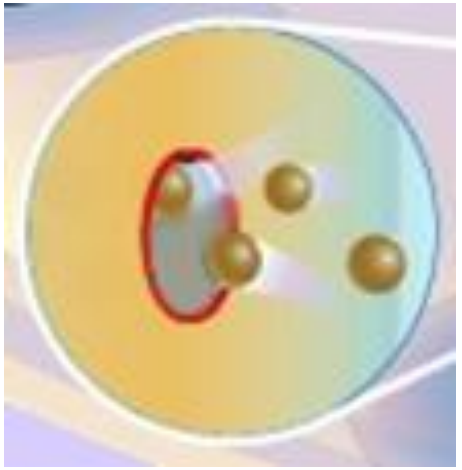
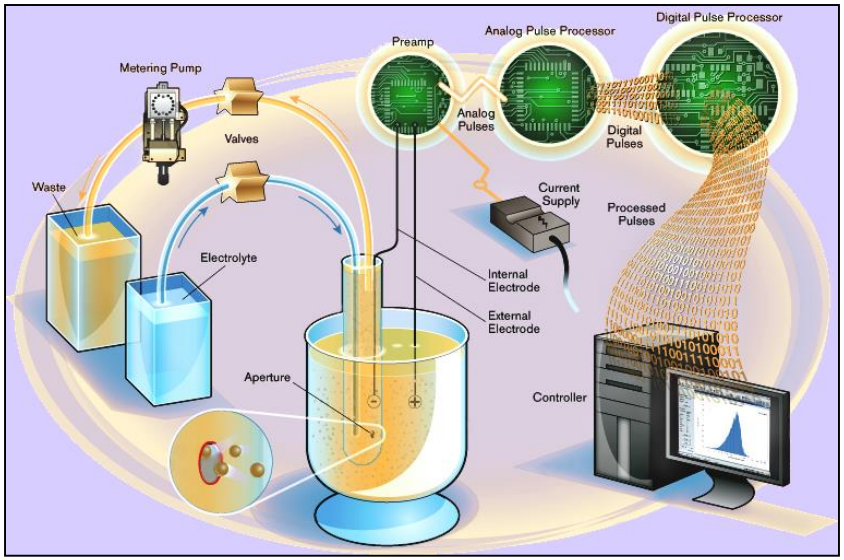
- Sophia Kenrick
- Sambit Kar
- Olga Laskina
- John Carpenter
- Richard Cavicchi
- Ellen Koepf
- Neil Lewis
- Rukman de Silva
- Dean Ripple
- USP expert committed for SbVP in biologics

Electrical Sensing Zone (Coulter Principle)







Adapted from Coulter