Examples related to the assessment of solubility/dissolution and particle size distribution

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Solubility and dissolution rate

- **Dissolution rate** is the critical element

  Under the anticipated use conditions the material will be fully dissolved in the marketed product, in food or, following ingestion, during the gastrointestinal tract processes: demonstration that dissolution kinetics is rapid enough to achieve full solubilisation in the stomach or in the intestine before gastrointestinal uptake of the particles

  A simplified dissolution rate assay in water (with 85 mmol/L NaHCO$_3$ and 40 mmol/L NaCl, pH=7) is offered. If solubility is pH-dependent and the criterion is not achieved at pH=7, it can be demonstrated that the dissolution rate at pH=3 (pH=5 for infants), representing the stomach conditions, is sufficiently rapid to ensure full dissolution in the stomach

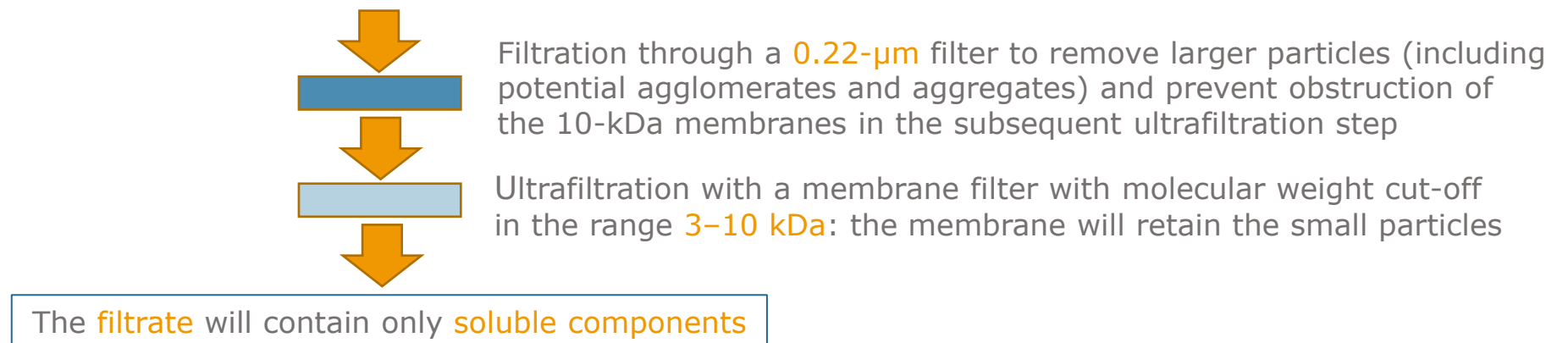- As a surrogate, **solubility threshold**: if very high solubility, dissolution is assumed to be sufficiently fast

  Solubility test according to OECD TG 105 or equivalent but removing any suspended particles from the suspension by ultrafiltration (ultracentrifugation and dialysis not to be used): solubility has to be equal to or higher than 33.3 g/L

Challenges with ultrafiltration

Ultrafiltration is the reference method to separate particulate and soluble fractions: as such, it is found in the (i) testing of solubility in water (2.3.1), (ii) assessment of dissolution rate in water (2.3.2), (iii) confirmation of absence of particles for liquid materials (2.3.3) and (iv) as a potential screening method for the presence of small particles and their size distribution (3.3.1.7 Filtration complemented with chemical analysis)

However, very large macromolecules represent a special case where the substance may be retained by the membrane during ultrafiltration even though it has no particulate nature

Sequential filtration helps in this case (see figure). If the issue is not solved, an argument may be put forward that the substance is not a particulate material and supported with adequate experimental evidence

Filtration through a 0.22-µm filter to remove larger particles (including potential agglomerates and aggregates) and prevent obstruction of the 10-kDa membranes in the subsequent ultrafiltration step

Ultrafiltration with a membrane filter with molecular weight cut-off in the range 3–10 kDa: the membrane will retain the small particles

The filtrate will contain only soluble components

Dispersion protocol
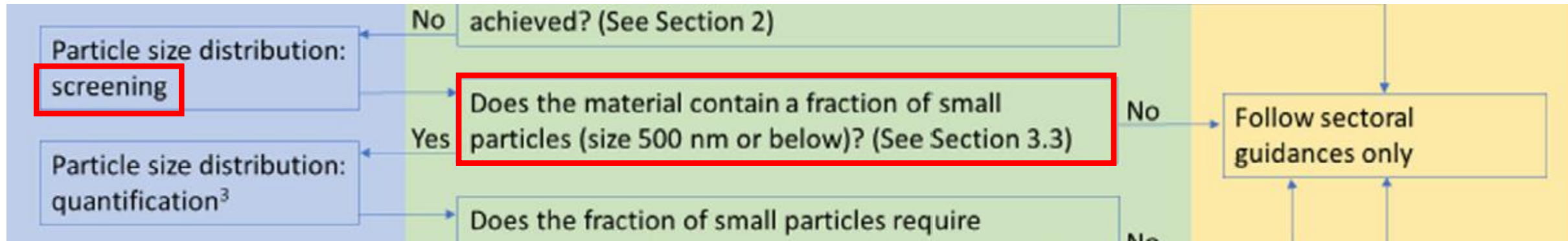
A proper sample dispersion is important for:

- **The assessment of dissolution rate in water (2.3.2)** - to deagglomerate potential agglomerates

- **Characterisation of the size distribution of the material including the fraction of small particles (3)** - for all measurements of particle size distribution a proper dispersion of the sample is **key** -> the recommendations presented in Section 3.2 on the "Dispersion protocol for sample preparation" have to be followed.

  The following general steps should be considered when developing a dispersion procedure:

  i) Choice of media, pre-dispersion and wetting can be the main limitations for an instrumental method used later

  ii) Choice of the method for deagglomeration/disaggregation of the material; the input energy used – i.e. is it sufficient to deagglomerate/disaggregate without changing the particle morphology. A general choice for most materials with a fraction of small particles is ultrasonic treatment using either a probe or vial sonicator

  iii) Stabilisation is the final and complex step in the process, therefore consideration of the choice of stabiliser is needed, along with other possible variables such as compatibility of the stabilisation method with the measurement method. Effectiveness of the stabilisation including the timescale for which stability must be ensured

- **Safety studies (4)** - the level of dispersion/degree of agglomeration of the test material has to be adequate for assessing the hazard of small particles including nanoparticles

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Adapted from TG, Fig. 1

Particle size distribution: screening	No — achieved? (See Section 2)	
	Does the material contain a fraction of small particles (size 500 nm or below)? (See Section 3.3)	No → Follow sectoral guidances only
Particle size distribution: quantification[3]	Yes	
	Does the fraction of small particles require	No

The applicant has the possibility to confirm using the *screening methods* that the material contains less than 10% of particles (number-based) with at least one dimension ≤ 500 nm;

Screening methods
- To demonstrate that less than 10% of the particles have at least one dimension ≤ 500 nm
- To give a positive indication of whether a given material contain small particles
- **Usually cannot** distinguish individual particles from aggregates or agglomerates
- **Cannot** be used to obtain a quantitative number based particle size distribution
- Screening EM: can give an indication of the material agglomeration/aggregation state
- Screening EM: can give an indication of the particle shape(s)

Screening methods listed in the TG are
- Centrifugal liquid sedimentation (CLS), Particle tracking analysis (PTA), descriptive electron microscopy, Filtration complemented with chemical analysis
- Other methods may be used with justification

Screening methods

The following methods **cannot be used** to screen the presence of small particles and characterize the particle size distribution of the material (3.3):

- **DLS** not suitable for particle size distribution, but may be relevant for checking the stability of dispersions

- **Laser diffraction** is generally unable to measure small- and nano-particles and is not a suitable method

- **VSSA** results cannot be related to the considered thresholds for particle size (500 nm and 250 nm) and thus the method can not be used

- Whatever is the method used, number-based particle size distributions have to be reported (issue of constituent particles vs agglomerates)

Screening methods – DLS (Dynamic Light Scattering) and LD (Laser Diffraction)

To keep in mind:

- **DLS** measures fluctuation of scattered light caused by Brownian motions of particles
- **LD** measures light scattered by particles (size from the diffraction pattern)
- Scatterers can be individual particles, aggregates or agglomerates

⚠ Many entities measured simultaneously (→ "ensemble method")

- **DLS**: hydrodynamic diameter of *equivalent spheres*
- **LD**: *diffraction equivalent spherical diameter*

Cannot be distinguished

⚠ ~~nonspherical particles~~

- **DLS:** Results are **light intensity-weighted**

As $I_{scattered} \sim r^6$ it gives much more weight to large particles. ($I(50\ nm) \approx 10^6\ (5\ nm)$)

- Conversion into number-weighted results is **not reliable**
- ⚠ **DLS alone cannot be used to prove the absence of small particles**

Further reading (for DLS):
- The NanoDefine Methods Manual. Part 2: Evaluation of methods, doi:10.2760/071877 (2019) -> overview
- Characterization of nanoparticles (ed. Unger et al.), Elsevier 2020, ISBN: 978-0-12-814182-3 -> in detail

DLS
- Bad size resolution
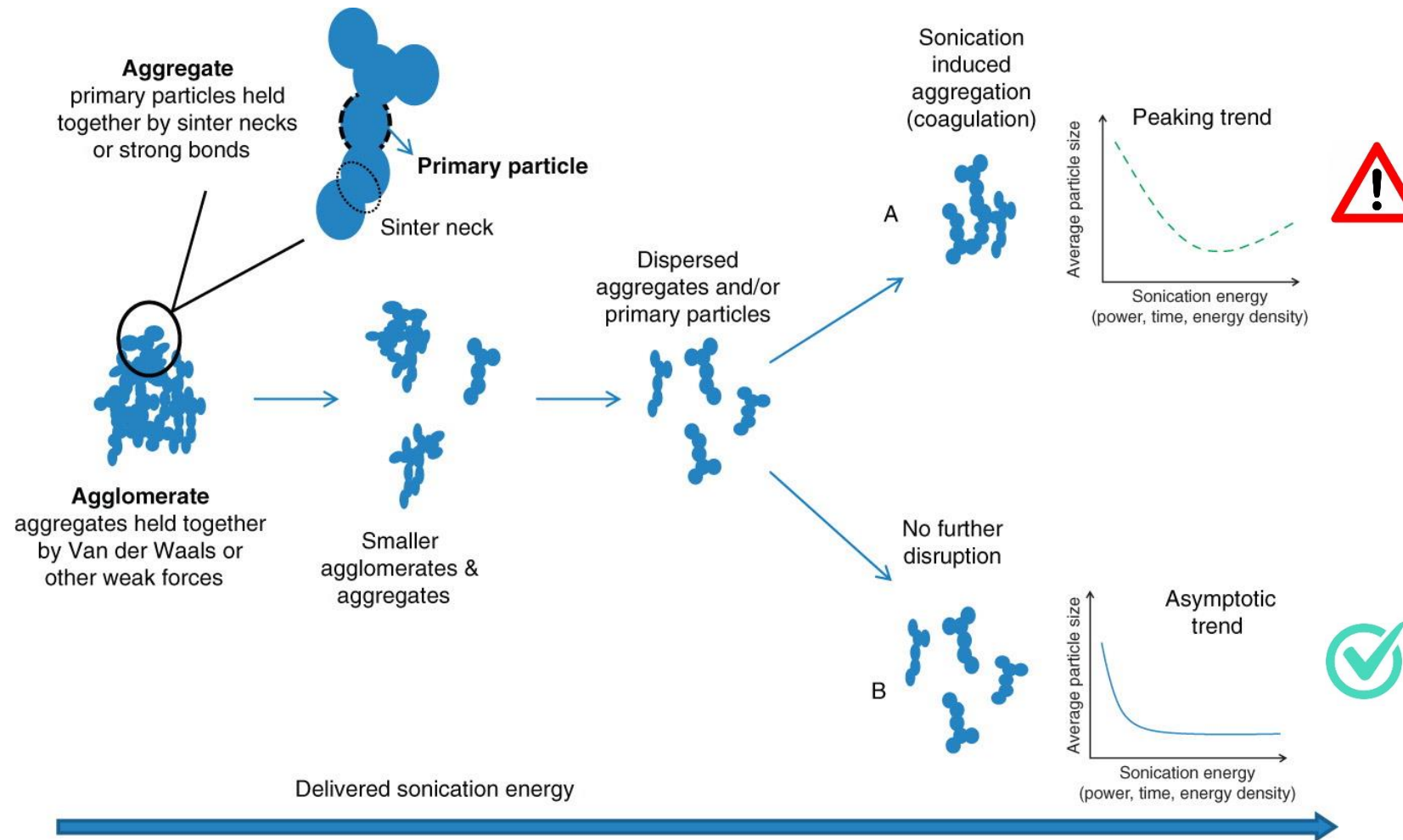- Larger particles dominate the signal

Individual suspensions: AuNP 5 nm, 15 nm, 45 nm
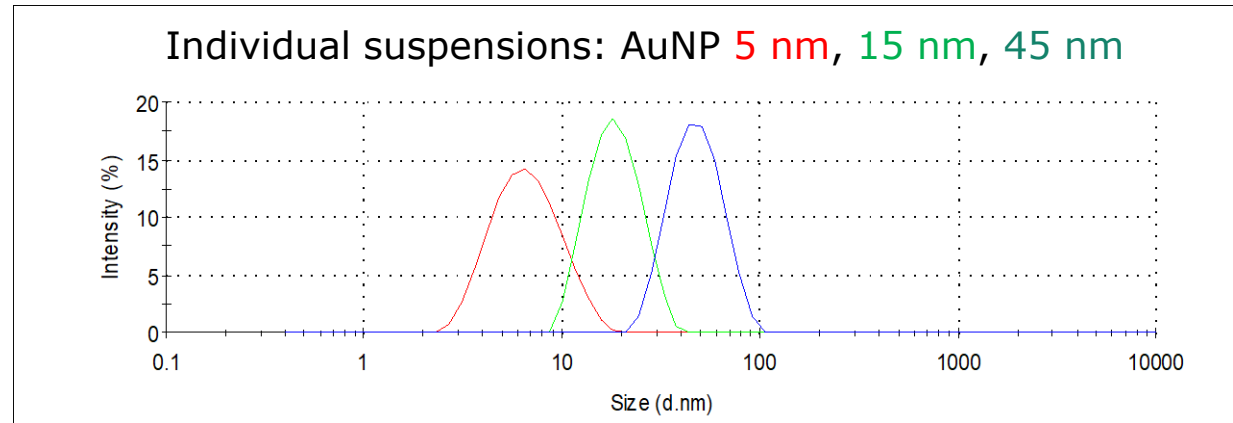
AuNP suspension mix 5 nm, 15 nm, 45 nm (1:1:1 in mass but **350:15:1 in numbers**)
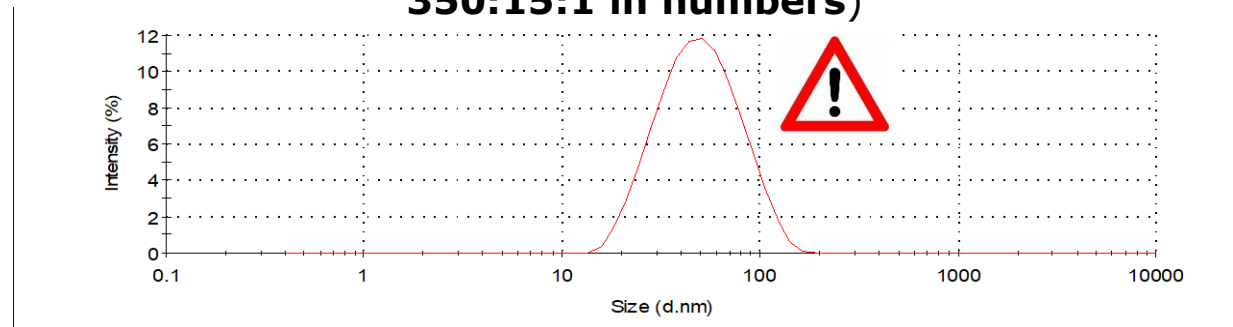
Basically similar distribution for mixture and pure 45nm NP!

Screening methods – DLS (Dynamic Light Scattering) for checking the stability of a dispersion

Instable dispersion due to sedimentation

Stable dispersion
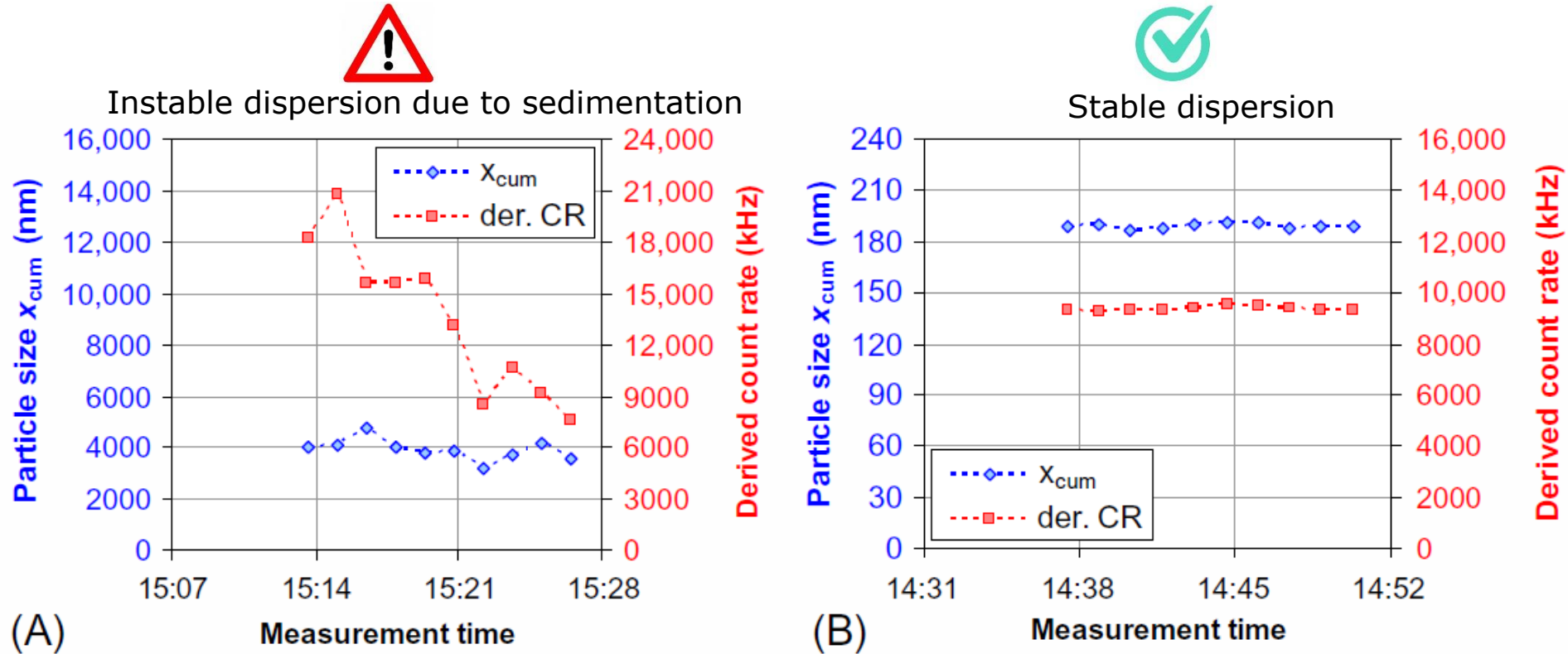
Fig. 9 Trend analysis for (A) significant sedimentation and (B) good sample stability; based on the effective hydrodynamic diameter (x_{cum}) and the total scattering intensity, which is quantified by the derived count rate (der. CR) of photons.

Note: if the measured particle size changes with **time** the dispersion is not stable (possibly) due to (i) sedimentation of larger particles or (ii) agglomeration

- Simple correlation of surface area with size only valid for truly spherical, non-porous, mono-modal, monodisperse, non-aggregated particles (usually not the case for food grade materials)

- Does not provide particle number/size distributions

- Not suited to exclude materials from consideration
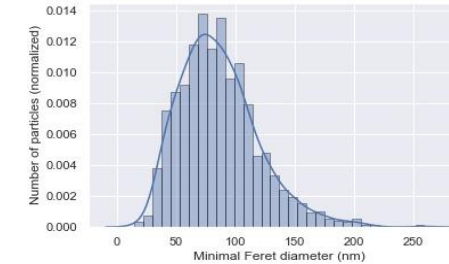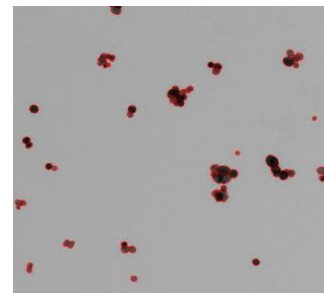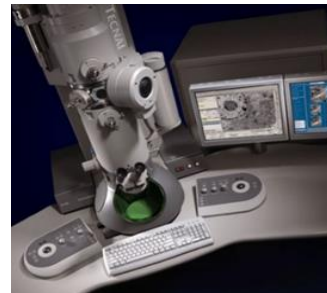
Reporting of the results of EM analysis

Sample
Preparation

Specimen
Preparation

EM
Imaging

Image
Analysis

Data
Analysis

- Objectives
  - Report clearly presents how the work (flow) is realized.
  - Measurement results of a representative fraction estimate the CP particle properties accurately and precisely.

Sample preparation

Sample Preparation → Specimen Preparation → EM Imaging → Image Analysis → Data Analysis
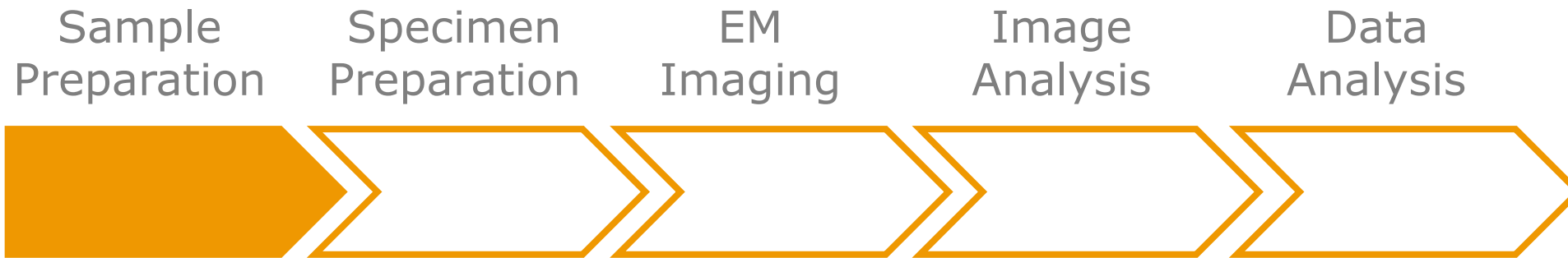
- **Objectives**
  - Stable dispersion
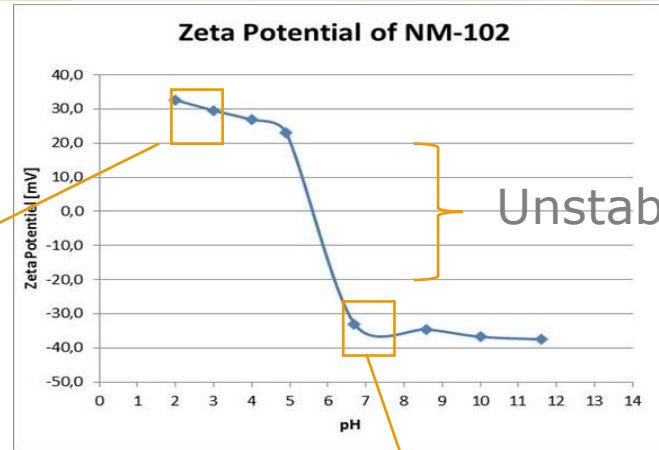 - Most dispersed state: deagglomeration

- **Protocol**
  - Nanogenotox, Nanoreg
  - Spalla & Guiot protocol
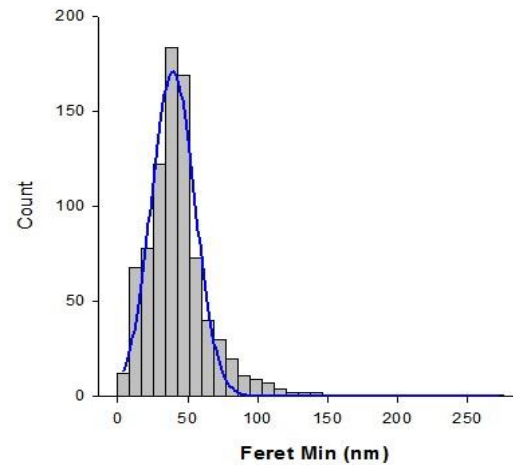 - MOSTLY: tailored for specific material
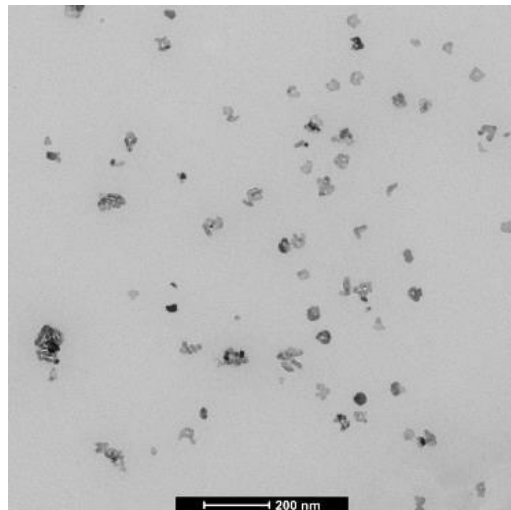
- **Principles**
  - Environmental conditions adapted to particle properties:
    - Polar - apolar medium, pré-wetting
    - pH adjustment based on ξ-potential
    - Salt and proteins concentrations
  - Deagglomeration/deaggregation: sonication, pH adjustment
  - Electrosterical stabilisation: BSA, surfactants,…
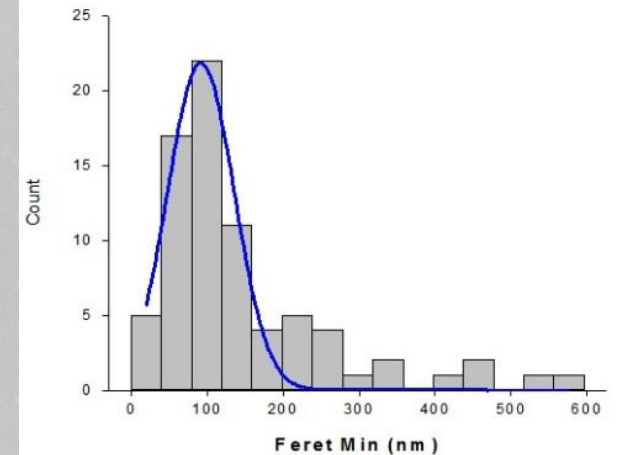 - Concentration: centrifugation
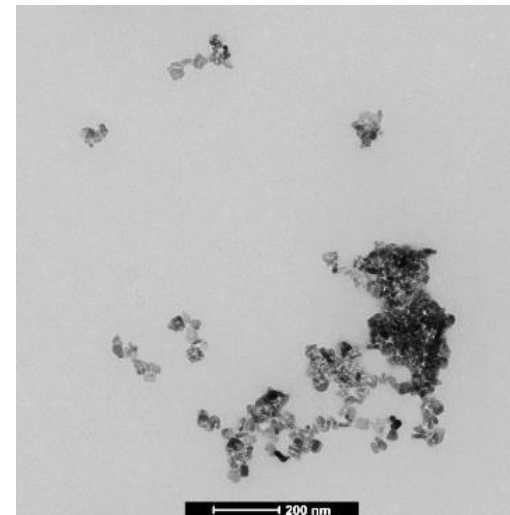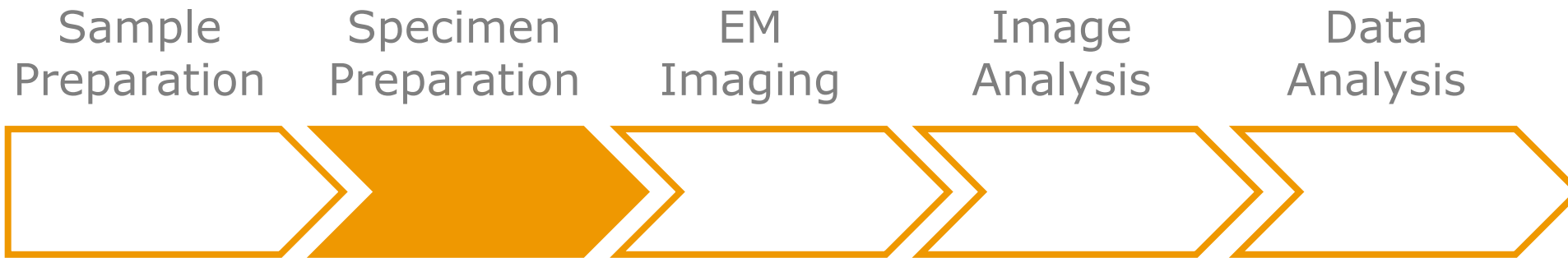
Example: Optimize pH

Zeta Potential of NM-102

Unstable in polar medium

pH 2 median Feret Min = 33 nm

pH 7 median Feret Min = 107 nm

EM specimen preparation

Sample
Preparation | Specimen
Preparation | EM
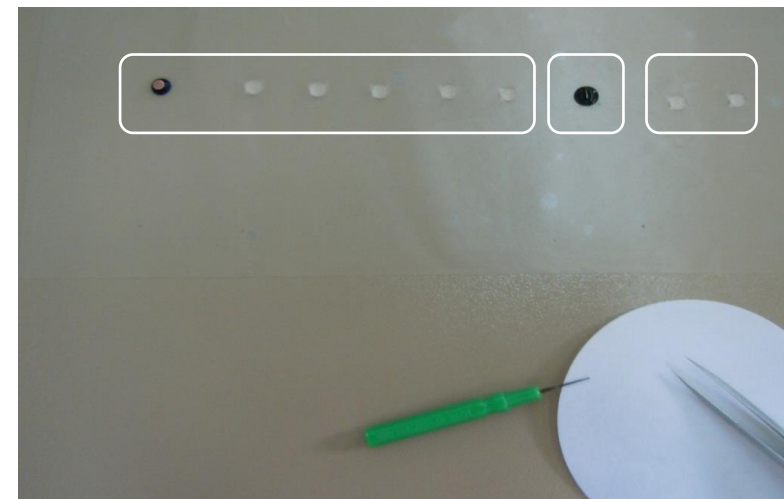Imaging | Image
Analysis | Data
Analysis

- **Objectives**

  ➢ Transfer of a representative fraction of particles to the support
  Uniform distribution and minimal overlap
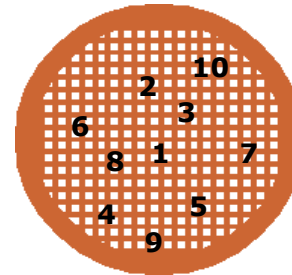 Free advise ☺ « Use EM grids, also for SEM! »

- **Protocol adapted to case**

  ➢ Grid on drop deposition (default)

  ➢ Drop on grid deposition (agglomerates)

  ➢ On-grid ultracentrifugation (conc. & quant.)

  ➢ UT sectioning (Preferential orientation platelets)

- **Principles**

  ➢ Grid charge compatible with particle charge (! ξ-potential)

  ➢ Deposition of dispersed particles followed by washing and drying

EM imaging

Sample Preparation → Specimen Preparation → **EM Imaging** → Image Analysis → Data Analysis

- **Objectives**
  - Representative micrographs + description (shows success of previous steps !)
    - Suitable for quantitative analysis (agglomeration)
    - Identification of relevant particles (purity)
    - Containing sufficient number of particles (conc.)

- **Imaging modes**
  - SEM: contrast, size, shape. Res > 10 nm !
  - TEM: contrast, size, shape. Res < 1 nm
  - STEM(-EDX): also chemical composition

- **Principles**
  - Systematic random imaging
  - Selection of magnification
  - = selection of pixel size
    = f(CCD camera * EM magnification)
    - LLOQ: Criterion of Merkus: smallest particle dimension ~ ϕ 10 pixels
    - ULOQ: 1/10 of image size (ISO 13322-1)
    - This determines the working range
  - Number of analyzed articles
    =f(measurement uncertainty)

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Image analysis

Sample Preparation → Specimen Preparation → EM Imaging → **Image Analysis** → Data Analysis

- **Software** – **protocol** - **algorithms**
- **Evolutions**
  - ➤ Increased performance and complexity
  - ➤ Automation
  - ➤ Operator → machine learning
- **Annotated images**
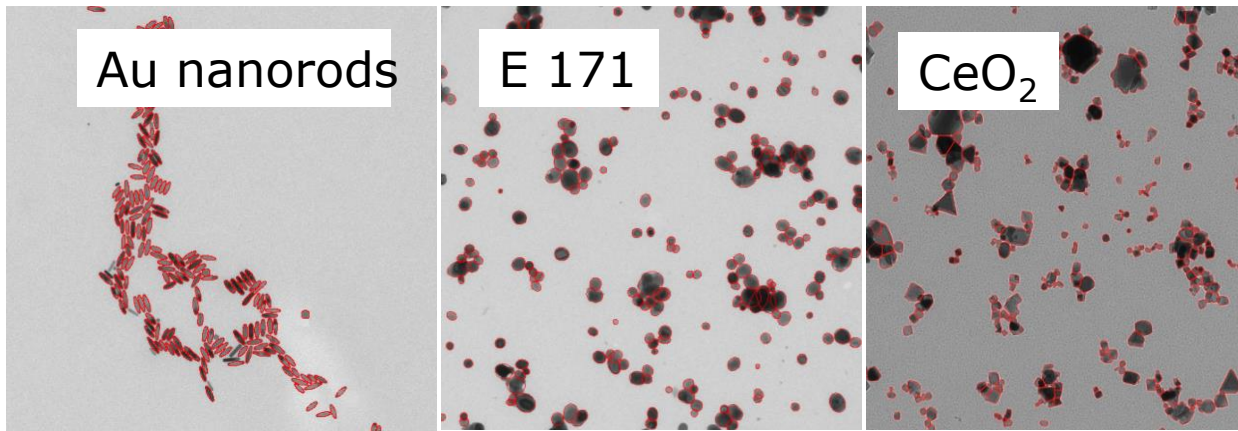

Au nanorods

E 171

CeO_2

Image pre-treatment
- Brightness & contrast
- Background
- Noise

Thresholding
- Grey-scale thresholding (manual or automated)
- Template matching
- Manual

Detection
- Region of interest
- Exclude border particles and artefacts
- Separation of (primary) particles

Measurement
- Simultaneous, multiple measurands
- Recording data in accessible format

Data analysis

| Sample Preparation	Specimen Preparation	EM Imaging	Image Analysis	Data Analysis

- ■ Technical guidance: intrinsic material property
  - ➤ number-based size distribution of CP (<500 nm)
  - ➤ Size parameter: minimum external dimension of CP, estimated by a suitable measurand (JRC Concepts and terms doi:10.2760/459136)
    - ➤ Minimum Feret diameter
    - ➤ Maximal inscribed circular diameter
    - ➤ Not ECD (unless spheres) or maximal Feret diameter
  - ➤ Descriptive statistics + Raw data
- ■ Nanoguidance: detailed characterisation for RA
  - ➤ Size, shape, surface topology, agglomeration, aggregation,…
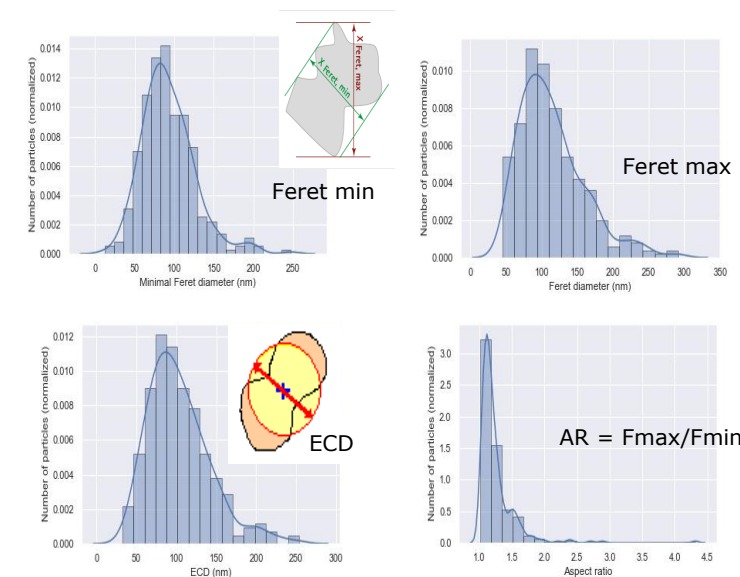
- Analytical methods used for size distribution (and solubility, dissolution rate) have to be validated (e.g. LOD/LOQ, precision, trueness; refer to Scientific Guidance)

- Measurement uncertainty
  - Needs to be determined from interlab/intralab precision data
  - Must be reported with each result
  - Extended measurement uncertainty (factor 2, relating to confidence interval of 95%) should be taken into account when considering the results with view to the postulated thresholds