Testing the test in NANoREG: Nanomaterial Characterization and Technical Guidance for Toxicological Testing

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NATIONAL RESEARCH
CENTRE FOR THE WORKING ENVIRONMENT

Outline

- **Brief general introduction to the EU FP7 NAN§REG project**
- **Introduction to WP2 Synthesis, supplying, and characterization**
  - Tasks
  - Regulatory questions addressed
  - Potential impacts
- **Select highlights of first results**
  - **Task 2.1: Identification of NM according to the EC definition**
  - **Task 2.4: Test item preperation, exposure, dose and fate for regulatory purposes and toxicology**
    - **NANoREG Technical Guidance Document**
    - **Minimum characterization Requirements**
- **End**

NANoREG - overview

Total budget ca. 50 Mio €
(ca. 67.5 Mio $); 20% from EU

Project duration: 42 Months
(started March 2013)

61 partners from
15 European countries

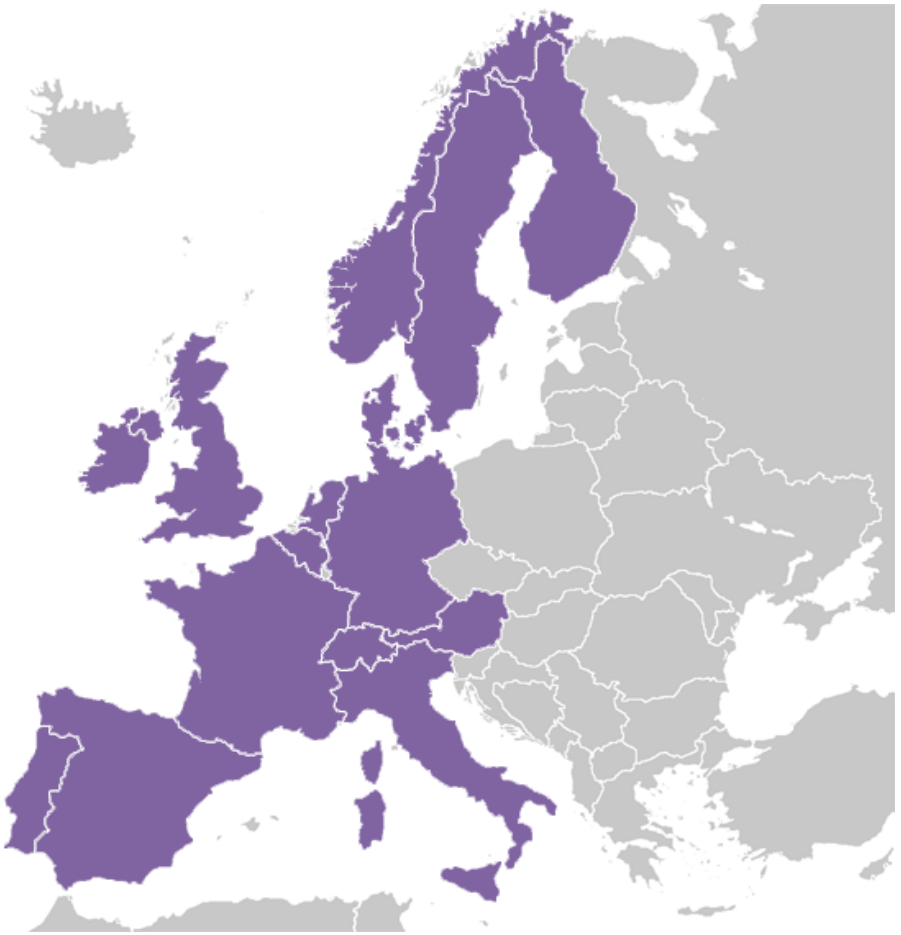
13 are EU member states
(AT, BE, DE, DK, ES, FI, FR, IR, IT, NL, PT, SE, UK)
2 associated states
(CH, NO),
and 1 PAN-EU JRC

Incoming:
Turkey, South Korea, Brazil

+ other „International“ collaboration

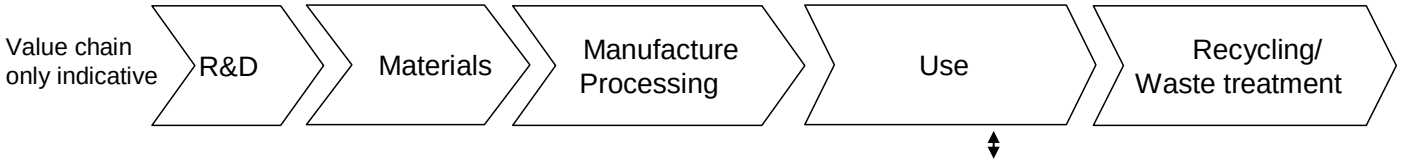
Coordinator: Tom van Teunenbroek
Ministry of the Environment, NL

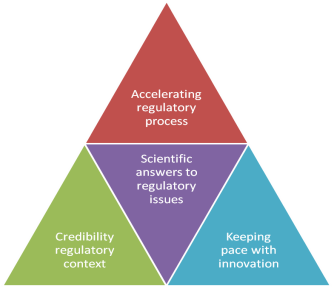
A common European approach to the regulatory testing of nanomaterials



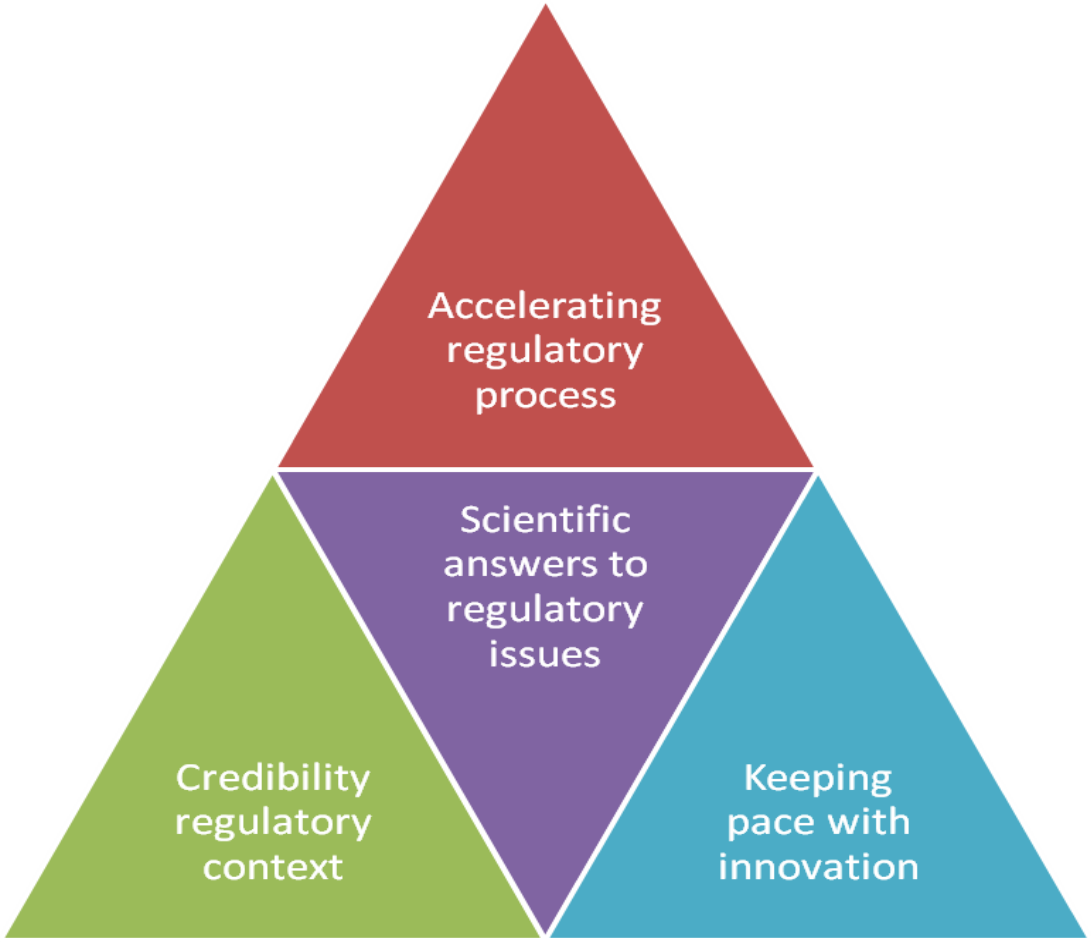
Project Key Information

- A project intended to **combine “all” the aspects of societal needs, innovation, exploitation & industry**
- Structured to **deliver answers on regulatory questions** coming from the **member states and organization** (e.g., OECD WPMNM)
- Specific focus will be on **the nanosafety methodology**
- Aim is to **identify, harmonize, and apply “reliable” methods** for characterization, testing, risk assessment and management
- Aim is to **establish a grouping paradigme for MNM based on phys-chem and toxicity** to enable faster, but still reliable risk assessment
- Lessons and demonstration will be made through NANoREG **Life-Cycle Value Chain Studies**

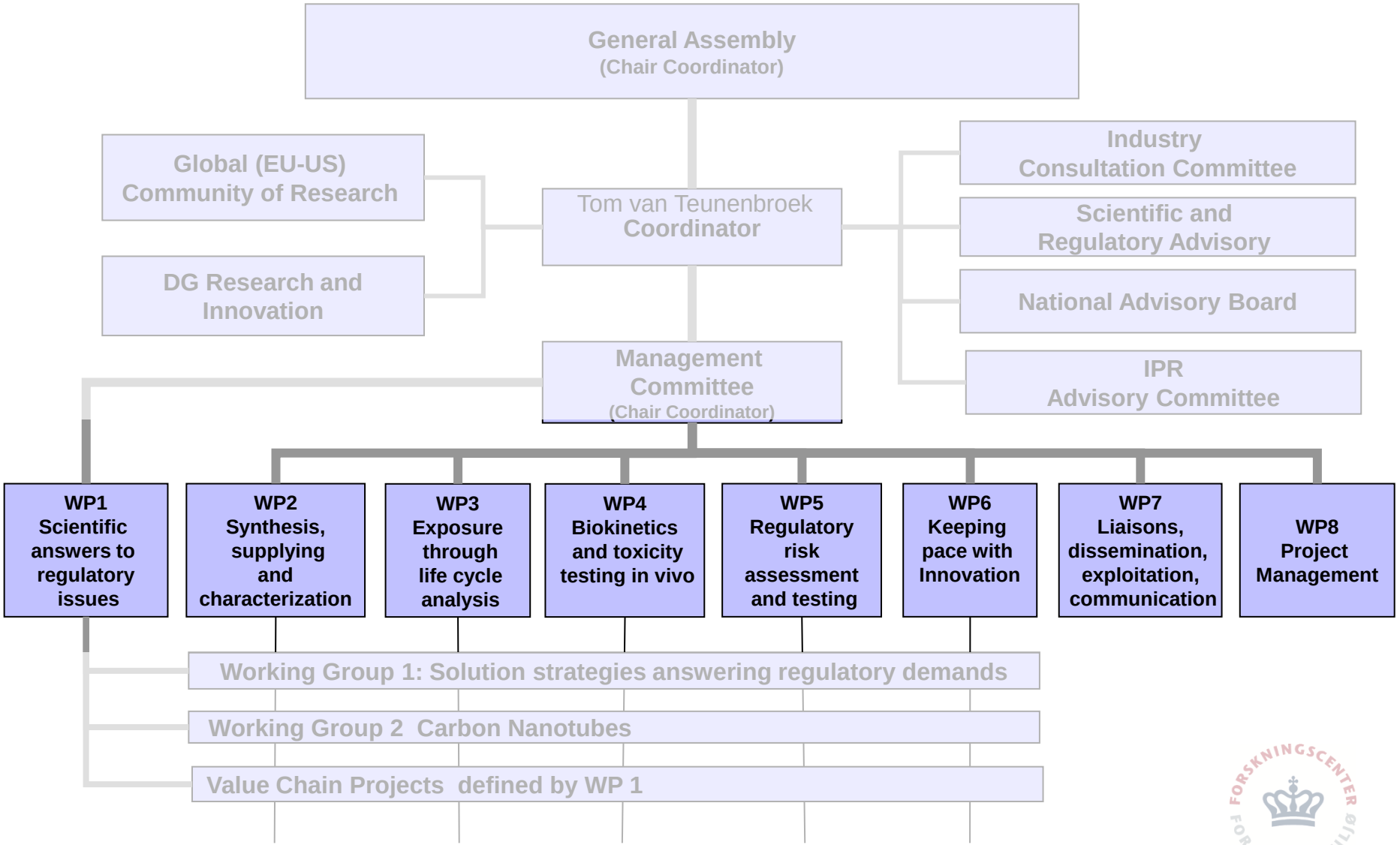




Key Objectives



NANoREG's Organisational Structure



WP2: Synthesis, supplying and characterization

Keld Alstrup Jensen (NRCWE, DK)

Main objectives of WP2

1) Synthesis and procurement

- availability and key characteristics of 19 core MNM (Total >80 MNM including additional 15 different CNTs)

2) Identification of MNM according to the EC regulatory definition

- number size-distribution, VSSA, MN categorization and nomenclature

3) NM Characterization SOPs for regulatory purposes

- SOPs supporting key OECD TGs and potential future methods

4) Test item preperation, exposure, dose and fate for regulatory purposes and toxicology

- technical guidance to WP3-WP5, benchmark values, methods and exposure characteristics in vivo inhalation, in vitro and ecotox studies

Core Manufactured Nanomaterials

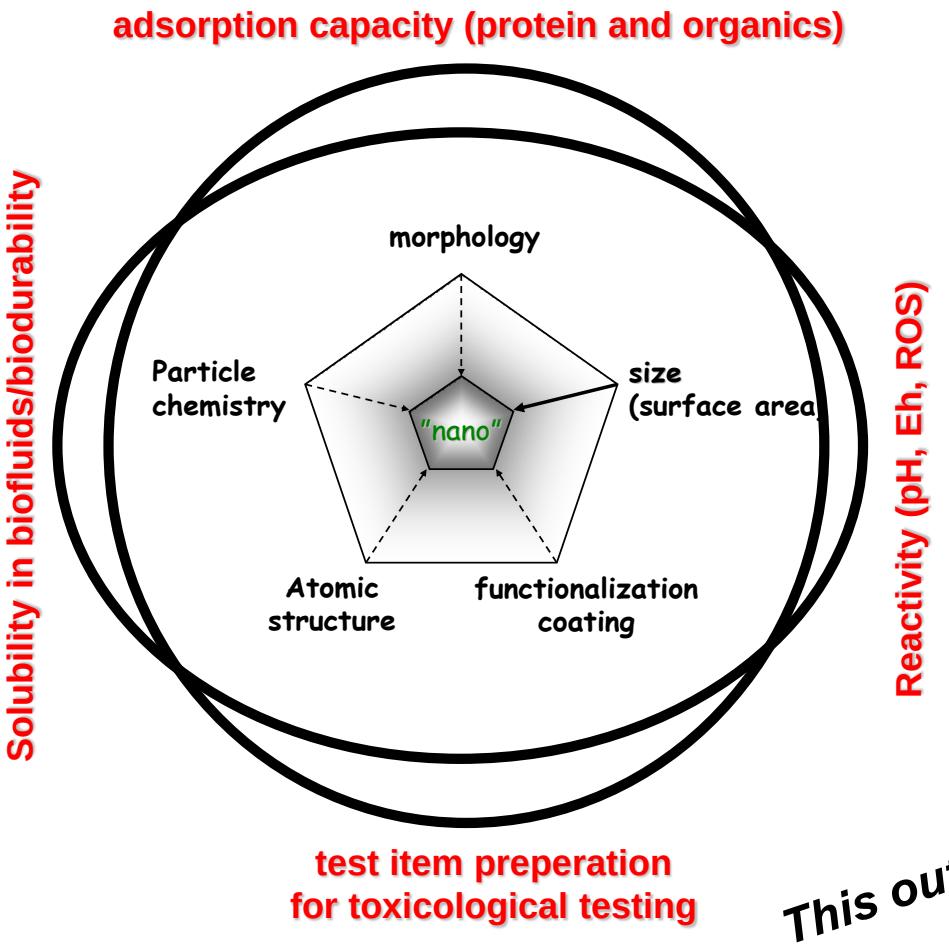
Type of MNM	MNM Identification codes used by NANoREG
Titanium Dioxide	NM101, NM102, NM103
Synthetic Amorphous Silica	NM200, NM203
Zinc Oxide	NM110, NM111
Cerium Dioxide	NM212
Barium Sulphate	NM220
Silver	NM300K, NM302
Nanotubes (single and multi-walled)	NM400, NM401, NM410
Nanofibrillar cellulose	NFC Fine, NFC Medium-coarse, UPM Biofibrils AS, UPM Biofibrils NS, UPM Bleached Birch Pulp
Final material closing knowledge gaps	Under evaluation

Key Regulatory Questions Addressed in WP2

- Measurements and characterization: Identification according to the EC definition; Applicability of OECD TG's
- Measurement and transformation: After entry into the body and the environment
- Metrology and dose metrics: Hazard, exposure, life-cycle assessment
- Extrapolation and grouping: Investigate read-across from bulk or grouping due to properties, exposure, mode of action
- Fate, persistence and long-term effects: Is there a link between bulk compounds and MNM
- Mode of action: Which PC properties affect biological systems and should be known for risk assessment?

- Measurement and characterization and transformation: Establishment of new potential characterization requirements for grouping and risk assessment.

Potential WP2 impact: SOPs for regulatory characterization needs



SOPs for EC definiton of MNM
SOPs for revision of OECD TGs
SOPs for new CEN or TGs
SOPs for test item preperation
Methods for in vivo testing
Methods for in vitro testing
Methods for ecotox testing

<u>Methods targeted</u>
Transfer to NANoREG WPs
OECD
CEN
ISO
……

This outcome makes regulation operational

We need also to understand the method-dependent differences and uncertainties!

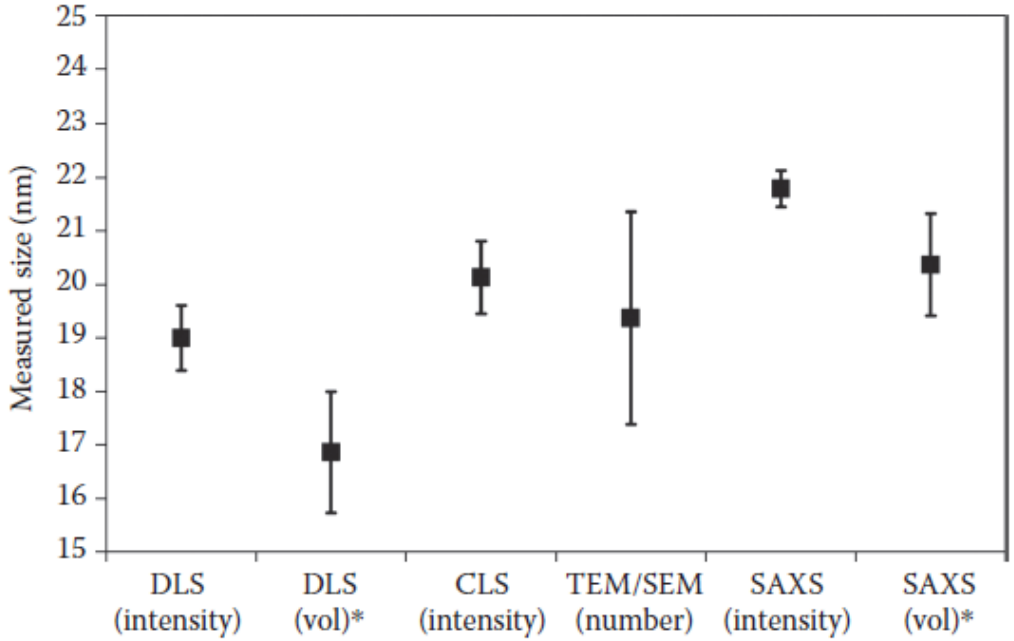


FIGURE 4.2 Size measurement data from the establishment of the certified reference material ERM-FD100 (spherical colloidal silica) using different measurement techniques and units applicable for the specific techniques: standard deviations include measurement uncertainty and interlaboratory variability from round robin test. The "*" denotes that the value in the specific unit is not certified.

Jensen et al. (2014) In Nanotoxicology: Progress Towards Nanomedicine, CRC Press

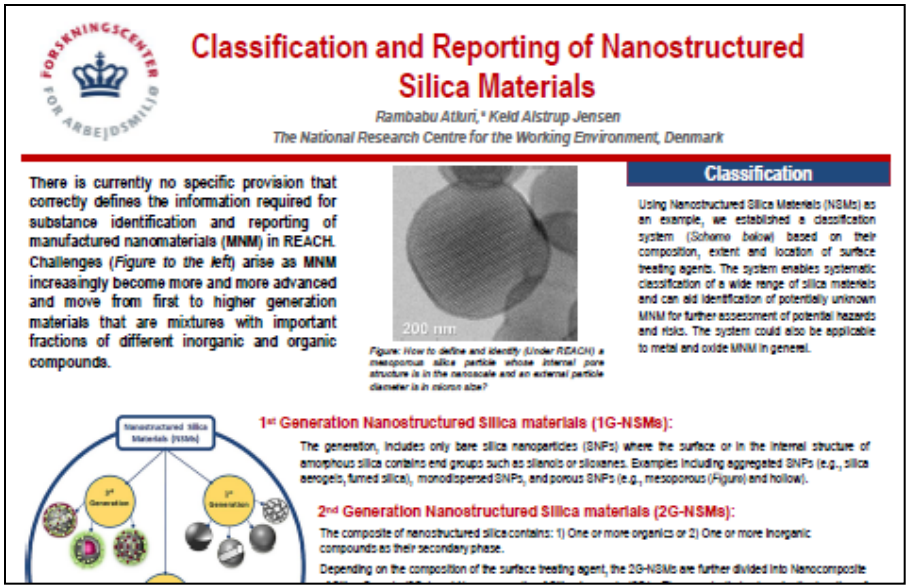
Select highlights of first results

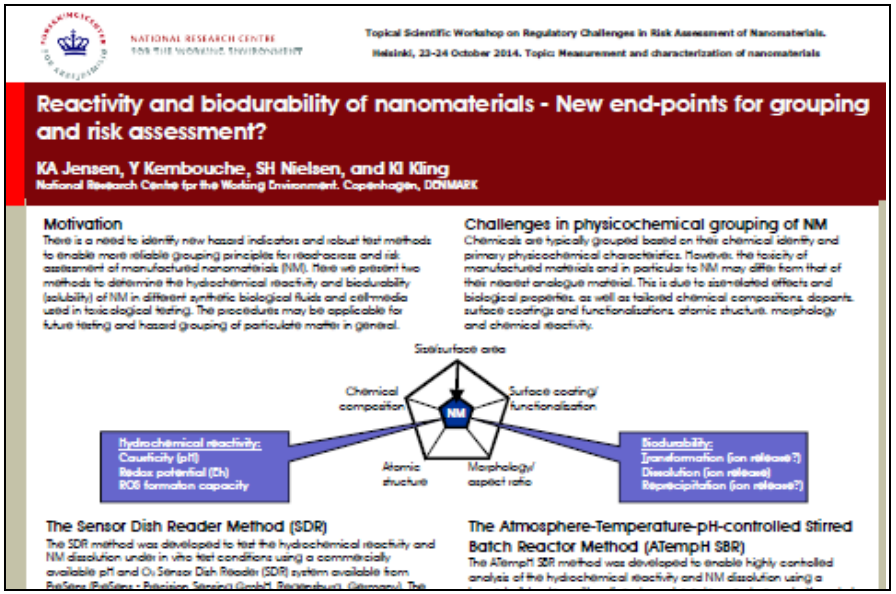
2) Identification of MNM according to the EC regulatory definition

- Number size-distribution, VSSA, MN categorization and nomenclature

4) Test item preperation, exposure, dose and fate for regulatory purposes and toxicology

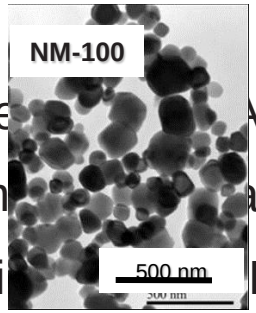
- Technical guidance to WP3-WP5, benchmark values, methods and exposure characteristics in vivo inhalation, in vitro and ecotox studies

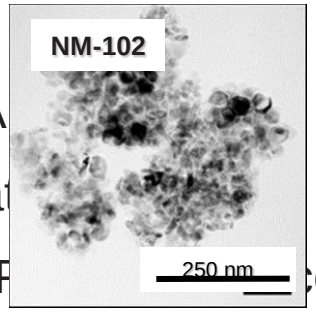


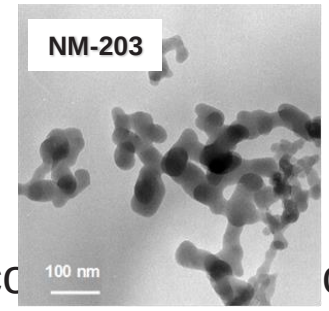


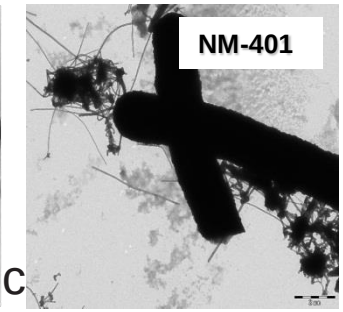
Task 2.2: Identification of MNM according to the EC regulatory definition

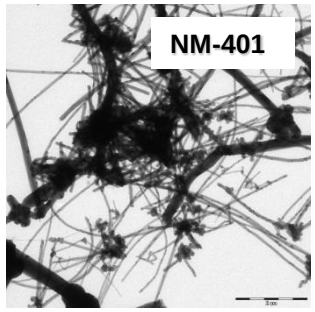
- **Number size-distribution**
  - TEM
  - Particle
  - Dynam
  - Scanni









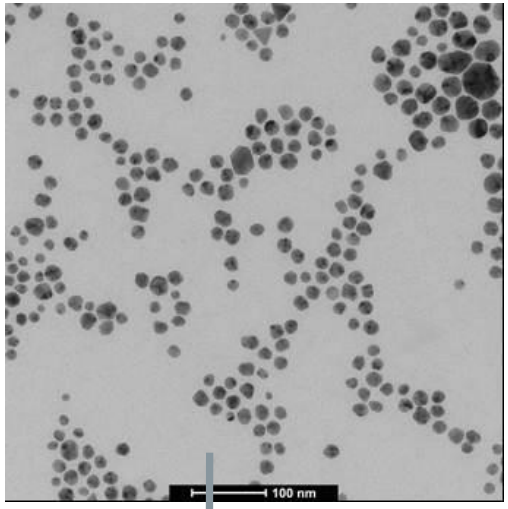


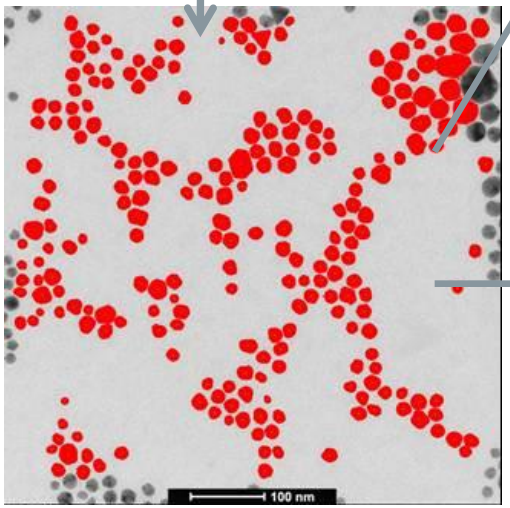
  - **Imaging mode: dependent on the complexity of the material and matrix:**
    - Simple matrix & pristine materials, at ingredient level: (conventional) BF-TEM:
    - Complex matrix & complex NM: STEM-EDX (coupled chemistry and imaging)
  - **Image analysis:**
    - Conventional: colloids, aggregates/agglomerates
    - Proof of principle: identification of primary particles in aggregates/agglomerates

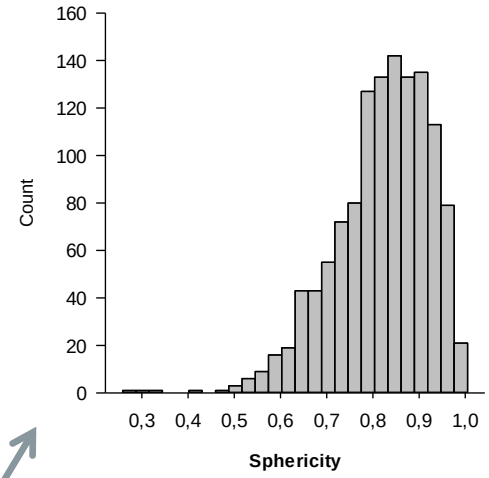
Source: De Temmerman, E Verleisen, J Mast (CODA CERVA)

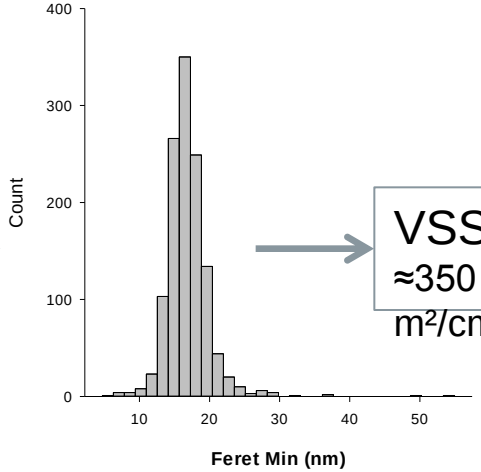
TEM characterisation of NM

Semi-automated measurement of physical particle properties



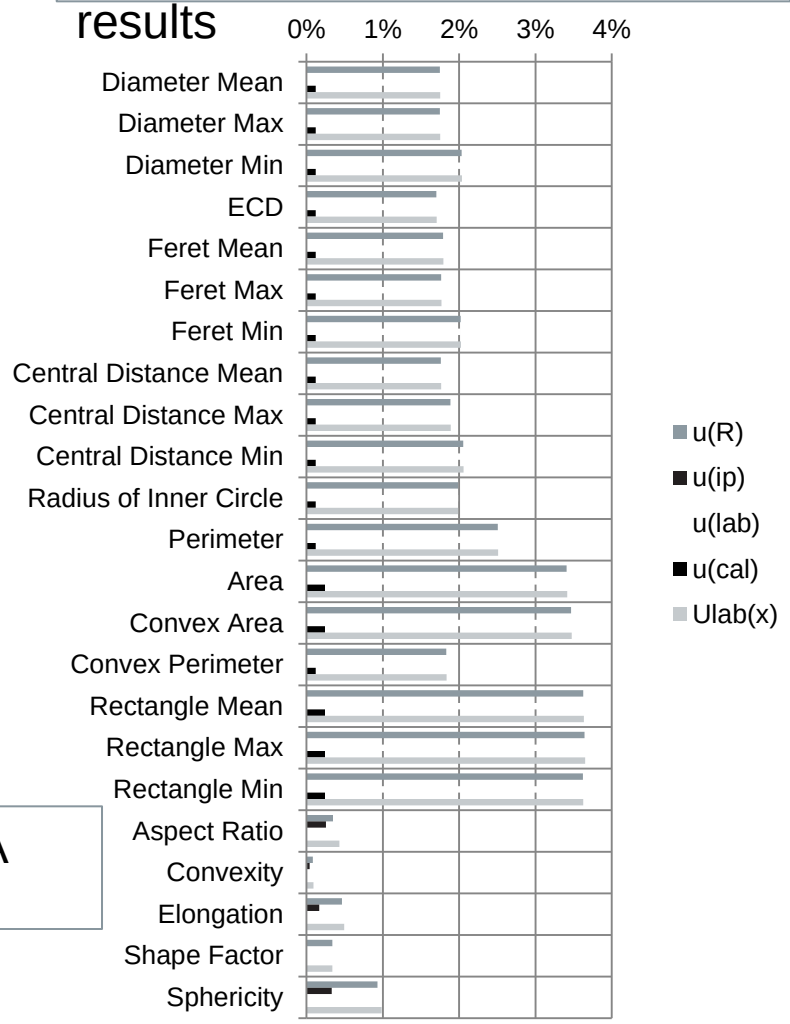






VSSA
≈350
m^2/cm^3

Validation of measurement results



Status of semi-automated TEM method:

Verified

- Colloidal
  - Gold NIST RM
  - SAS silica CRM
  - Ag (NM-30x)
  - Au (NM-33x)
- Powdered
  - TiO_2 (NM-10x)
  - ZnO (NM-11x)
  - SAS (NM-20x)
  - CeO (NM-21x)

Pending outcomes

- NANoREG
  - Colloidal
  - Mixtures and nanorods
  - Aggregates and complex NM
- NanoDefine
  - $CaCO_3$
  - Pigment yellow
  - $BaSO_4$
  - Difficulties expected for
    - Nanoplates like nanosteel
    - Kaolin

Person time required for analysis:
Semi-automatic: ca. 120 minutes
Automated: ca. 40 minutes

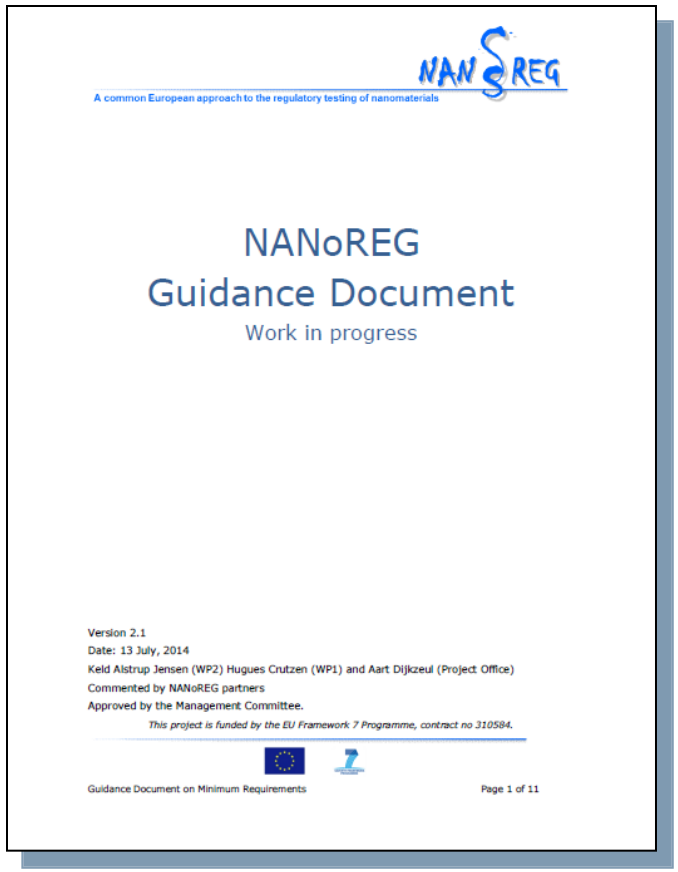
Derivation: > 20 size parameters and VSSA

- **Recording TEM micrographs**
- **Storing micrographs in database**
- **Analysing images**
- **Processing data**
- **Reporting**
  - Descriptive statistics
  - Number-based distributions

Source: De Temmerman, E Verleisen, J Mast (CODA CERVA)

Task 2.4: Test item preperation, exposure, dose and fate for regulatory purposes and toxicology

- **The NANoREG Technical Guidance Document**



- ✓ **Which MNM to test**
- ✓ **SOPs for selected dispersion and probe-calibration**
- ✓ **Benchmark data on batch dispersions**
- ✓ **Minimum characterization requirements in the toxicological studies**
- ✓ **SOPs for DLS measurement, sample preperation, qualitative TEM analysis**
- ✓ **Reporting requirements to NANoREG data-base**

Aims of the Technical Guidance Document

Probe-sonicator calibration protocol

Harmonize de-agglemeration energies/efficiencies

Batch dispersion protocols

Harmonize Initial Exposure Characteristics (per protocol)

In vivo | In vitro | ecotoxicology

Exposure characterization methods and protocols

Harmonized reporting ease check of comparability between tests

Interpretation, interpolation, extrapolation, read-across ….

Dispersion protocols

Type of test	Protocol
Calibration of sonicators for in vitro and in vivo studies	Calorimetic method combined with adjustment using the NM200 benchmark material NANOGENOTOX batch medium
In vitro studies	NANOGENOTOX
In vivo studies	NANOGENOTOX or ENPRA
Calibration of sonicators for ecotox studies	Calorimetic method combined with adjustment using the NM200 benchmark material in water
Eco-toxicity studies	A NANoREG water and a NOM*-water protocol for CNT

* Natural Organic Matter

- **Probe-sonicator calibration protocol developed in collaboration with NANODEFINE and based on Taurazzi et al. 2012 (NIST procedure)**

Probe-sonicator dispersion protocols (ca. 7.35 Watt at low amplitude)

- **NANOGENOTOX (Jensen et al. 2011) 0.5 v/v% EtOH and 0.05% w/v Albumin**
- **ENPRA (Jacobsen et al., 2010) 2% serum water**
- **Water and NOM protocols in accord with developments in OECD**

Please contact me if you want further information in the protocols

Characterization requirements

Element in the workflow	Recommendation (R) and Mandatory requirement (M); Optional (O)
Nanomaterial check	(R)
Batch dispersion	Ten repeated measurements of hydrodynamic size (DLS) are made without pause in combination with verification or measurement with TEM, SEM or AFM which-ever is most suitable. In vitro (M) and eco-tox (M).
Initial exposure medium	Ten consecutive measurements of hydrodynamic size (DLS) are made (if technically possible) without pause on the same sample in combination with verification or measurement with TEM, SEM or AFM which-ever is most suitable. In vitro (M) and eco-tox (M)
Final exposure medium	Ten consecutive measurements of hydrodynamic size (DLS) are made (if technically possible) without pause on the same sample in combination with verification or measurement with TEM, SEM or AFM which-ever is most suitable. In vitro (M) and eco-tox (M).
Stability of dispersion during assay	(R)
Contextual conditions and reactivity in the during testing	Measure several of the following parameters (pH, T, conductivity, redox potential and the CO_2/O_2 concentrations) during testing. In vitro (R) and eco-toxicity (M).
Dissolution in batch dispersion and test media[€]	(R)

Why DLS as the common tool?

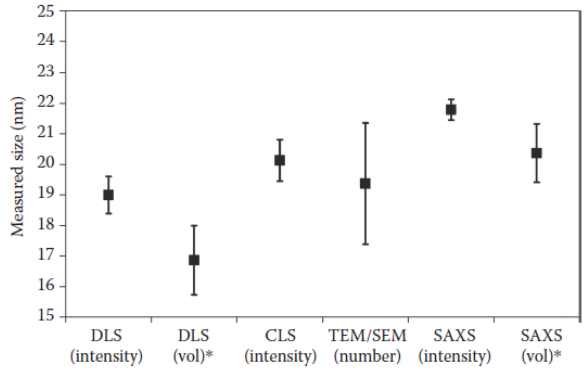
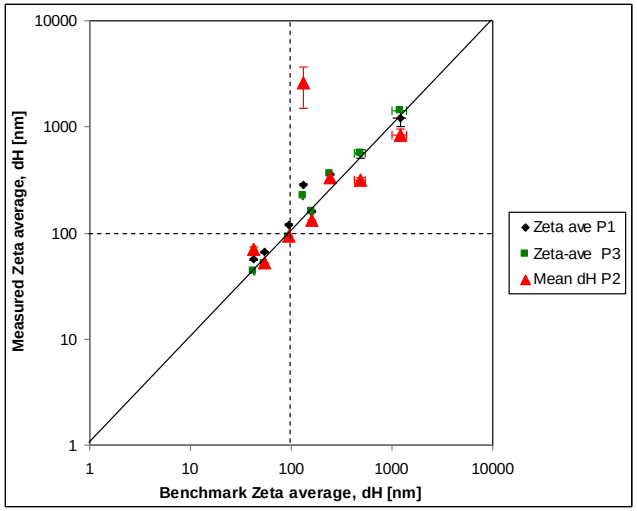


FIGURE 4.2 Size measurement data from the establishment of the certified reference material ERM-FD100 (spherical colloidal silica) using different measurement techniques and units applicable for the specific techniques: standard deviations include measurement uncertainty and interlaboratory variability from round robin test. The "*" denotes that the value in the specific unit is not certified.

Less user-depency and highly sensity to general changes in dispersion quality
Widely accessible, time and ease of use, instrument-derived values.

Jensen et al. (2014) In Nanotoxicology: Progress Towards Nanomedicine, CRC Press

- **Experience from previous projects (here NANOSUSTAIN) also generally show high comparabiloty between analysis of dispersions in different laboratories**

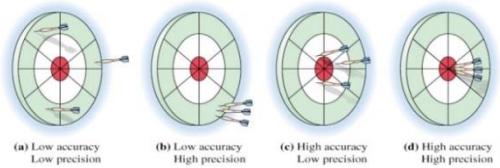


Is this type of guidance characterization and harmonization and really needed?

Yes!

(at least for now)

Is such extensive characterization really needed?



- **Know what you test!**
  - Verify or generate the PC data needed to understand the test material
- **Proper PC data will/may form the foundation for read across and hazard model development**
  - Reliable links between the NM properties and their (mechanism of) toxicological effects (e.g., empirical, ADME or QSAR-like models)
- **Understand the exposure characteristics**
  - Needed to interpret the toxicological test results (e.g. role of stability)
  - Reliable links between the NM properties and their (mechanism of) toxicological effects (e.g., empirical, ADME or QSAR-like models)

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So now we are Ready to Test the Test

Thank You for Your Attention

Remember to visit the posters

Identification of MNM according to the EC regulatory definition

- Number, Size by smallest dimension, and SSA are key nano-specific parameters
  - Scale and ISO definition (except 1 nm vs. ca. 1 nm)
  - Size and SSA are generally more hazard related parameters than mass for particle exposure (numerous studies)
  - Size and SSA may even be two of the parameters relevant for grouping and read-across principles
- The number fraction can be applied in readily dispersive granular, flaky, elongated, fibrous, and tubular materials (definition specifies the shortes dimension)
  - Size and percentage limits are political decisions (SCHENIHR, 2010)
  - The parameter is always true within the accuracy and precision of the applied techniques
  - Suitable sample preparation is key to obtain fully reliable results (best dispersion medium (solubilization) and sonication)
  - SOP for analysis by TEM has been completed and is the first choice for non-platy materials (NANoREG semi-automatic procedure approaches 2 hours per prepared sample, full automatization is on the way with ca. 40 min analytical time per sample)
  - AFM is a strong candidate for platy materials (not tested in NANoREG)
  - Specific near-1-nm compounds such as fullerenes, SWCNT, graphene, dendrimers, quantum dots etc. (and others to be added in the future) can be analysed by material specific techniques, even PCS, if any of the above would not be suitable (some are included as NM by definition e.g., fullerene and would not need to be analysed),
- The VSSA approach is generally a suitable supporting alternative approach where the 60 m^2/cm^3 is a spherical equivalent to 100 nm.
  - Limit is a political decision
  - The procedure appears generally not to be too overprotective becuase the size-distribution skews SSA power downwards for monomodal distributions
  - SOP has established for the BET nitrogen adsorption method and is under testing
  - Inclusion of relative density using pycniometry will be completed
  - System does not always hold as a filter (some NM fall out) so it should not be used as a screening tool alone.

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