

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

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NATIONAL RESEARCH  
CENTRE FOR THE WORKING ENVIRONMENT

A common European approach to the regulatory testing of nanomaterials



# 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 preparation, 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**

**Coordinator: Tom van Teunenbroek  
Ministry of the Environment, NL**



*A common European approach to the regulatory testing of nanomaterials*

**Project duration: 42 Months  
(started March 2013)**

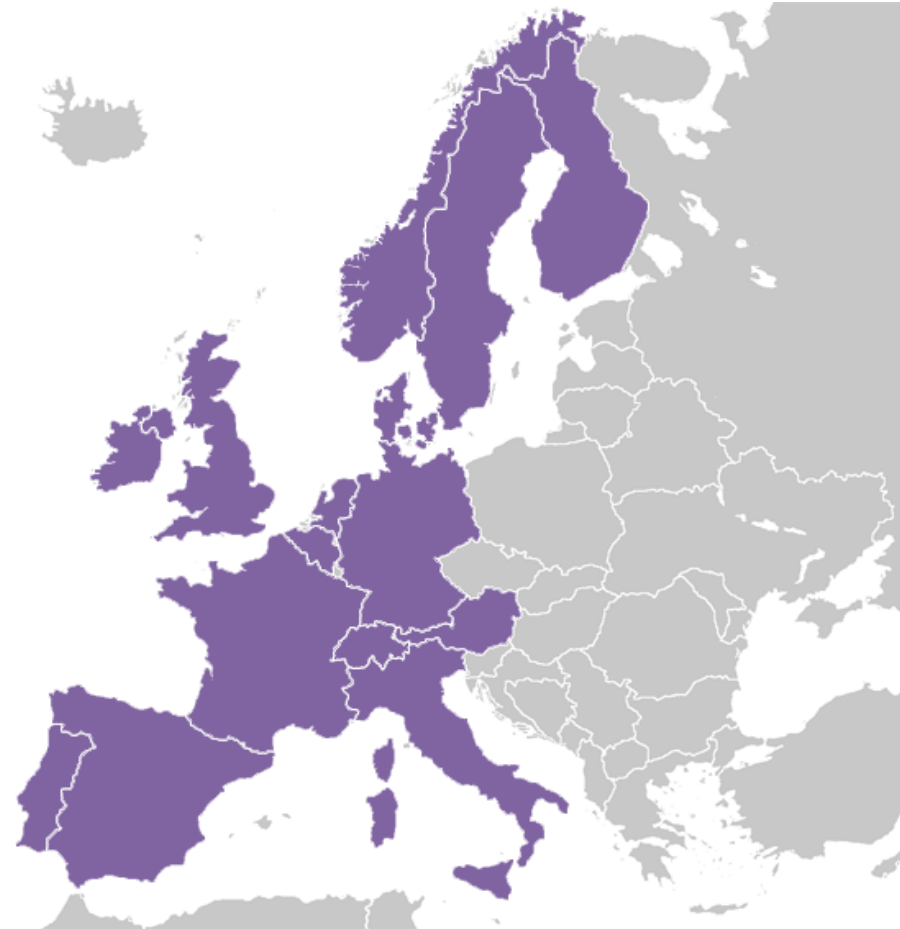
**61 partners from  
15 European countries**

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

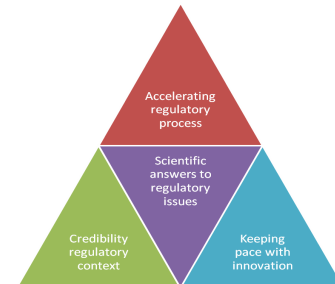
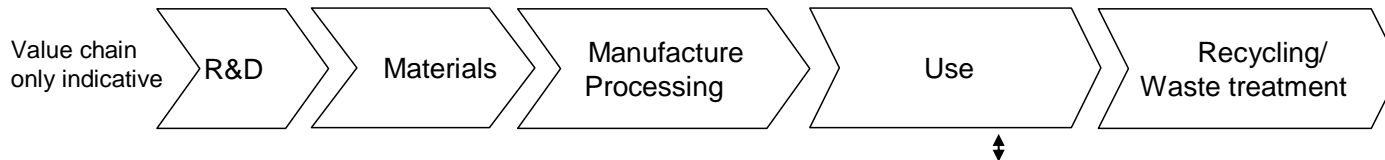
Incoming:  
Turkey, South Korea, Brazil

**+ other „International“ collaboration**

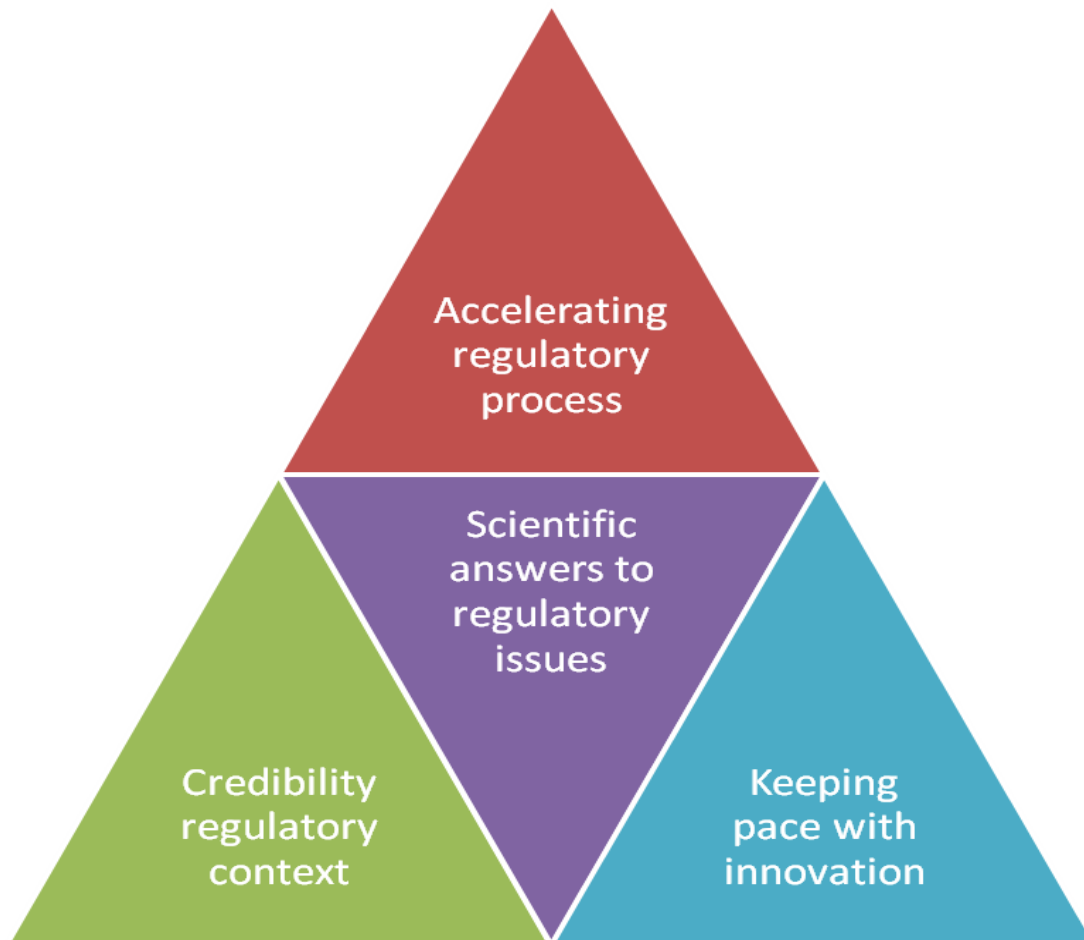


# Project Key Information

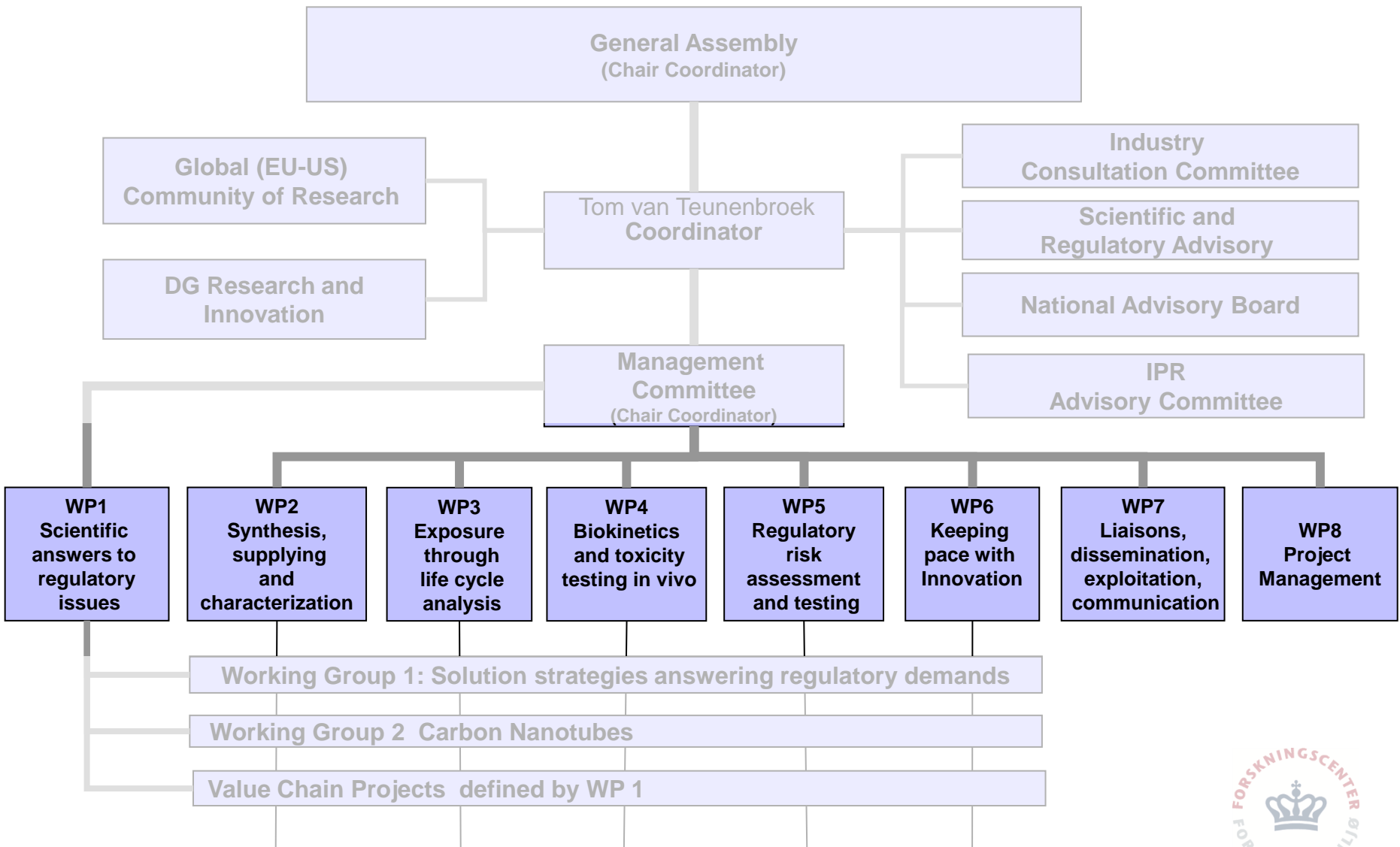
- A project intended to **combine “all” the aspects of societal needs, innovation, exploitation & industry**
- Structured to **deliver answers on regulatory questions** coming from the **member states and organization** (e.g., OECD WPMNM)
- Specific focus will be on **the nanosafety methodology**
- Aim is to **identify, harmonize, and apply “reliable” methods** for characterization, testing, risk assessment and management
- Aim is to **establish a grouping paradigm 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 preparation, 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
<b>Titanium Dioxide</b>	NM101, NM102, NM103
<b>Synthetic Amorphous Silica</b>	NM200, NM203
<b>Zinc Oxide</b>	NM110, NM111
<b>Cerium Dioxide</b>	NM212
<b>Barium Sulphate</b>	NM220
<b>Silver</b>	NM300K, NM302
<b>Nanotubes (single and multi-walled)</b>	NM400, NM401, NM410
<b>Nanofibrillar cellulose</b>	NFC Fine, NFC Medium-coarse, UPM Biofibrils AS, UPM Biofibrils NS, UPM Bleached Birch Pulp
<b>Final material closing knowledge gaps</b>	Under evaluation



# Key Regulatory Questions

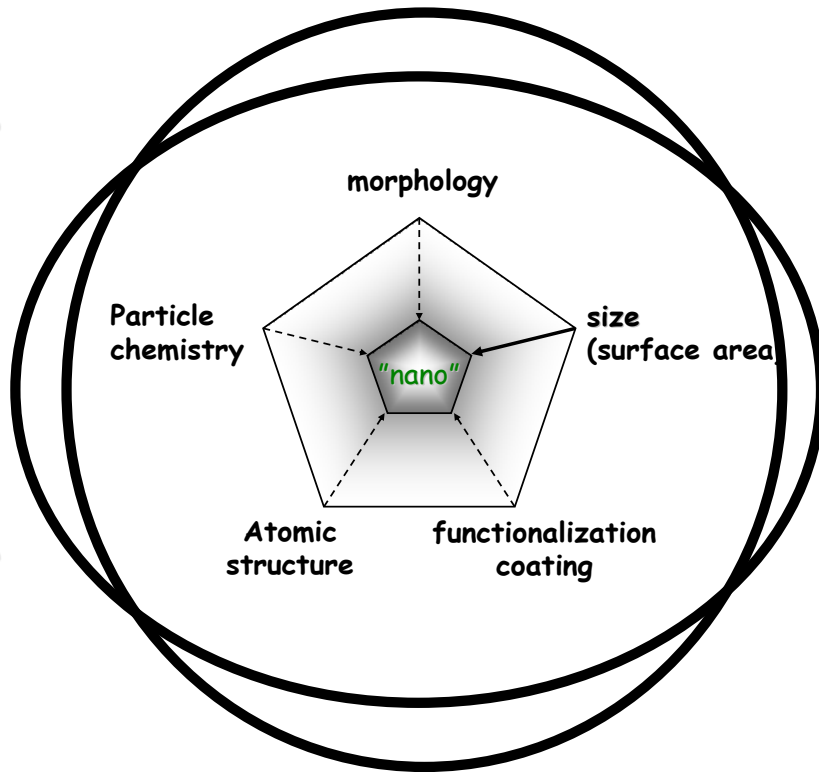
## Addressed in WP2

- 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

adsorption capacity (protein and organics)

Solubility in biofluids/biodurability



Reactivity (pH, Eh, ROS)

test item preparation  
for toxicological testing

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

## Methods targeted

Transfer to NANoREG WPs

OECD

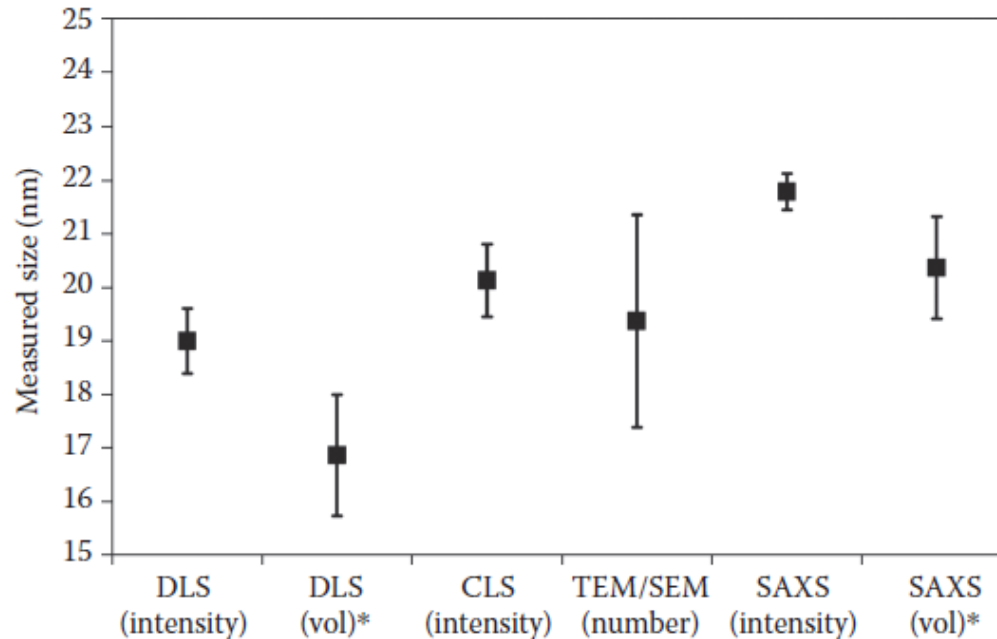
CEN

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.



# 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 preparation, exposure, dose and fate for regulatory purposes and toxicology

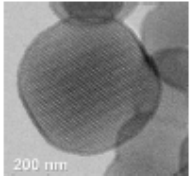
- Technical guidance to WP3-WP5, benchmark values, methods and exposure characteristics in vivo inhalation, in vitro and ecotox studies

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### Classification and Reporting of Nanostructured Silica Materials

Rambabu Atluri,\* Keld Alstrup Jensen  
The National Research Centre for the Working Environment, Denmark

There is currently no specific provision that correctly defines the information required for substance identification and reporting of manufactured nanomaterials (MNM) in REACH. Challenges (Figure to the left) arise as MNM increasingly become more and more advanced and move from first to higher generation materials that are mixtures with important fractions of different inorganic and organic compounds.



200 nm

Figure: How to define and identify (Under REACH) a mesoporous silica particle whose internal pore structure is in the nanoscale and an external particle diameter is in micron size?

#### Classification


Using Nanostructured Silica Materials (NSMs) as an example, we established a classification system (Scheme below) based on their composition, extent and location of surface treating agents. The system enables systematic classification of a wide range of silica materials and can aid identification of potentially unknown MNM for further assessment of potential hazards and risks. The system could also be applicable to metal and oxide MNM in general.

#### 1<sup>st</sup> Generation Nanostructured Silica materials (1G-NSMs):

The generation includes only bare silica nanoparticles (BNPs) where the surface or in the internal structure of amorphous silica contains end groups such as silanols or siloxanes. Examples including aggregated BNPs (e.g., silica aerogels, fumed silica), monodispersed BNPs, and porous BNPs (e.g., mesoporous (Figure) and hollow).

#### 2<sup>nd</sup> Generation Nanostructured Silica materials (2G-NSMs):

The composite of nanostructured silica contains: 1) One or more organics or 2) One or more inorganic compounds as their secondary phase.  
Depending on the composition of the surface treating agent, the 2G-NSMs are further divided into Nanocomposite



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NATIONAL RESEARCH CENTRE FOR THE WORKING ENVIRONMENT  
TOPICAL SCIENTIFIC WORKSHOP ON REGULATORY CHALLENGES IN RISK ASSESSMENT OF NANOMATERIALS  
HELSINKI, 23-24 OCTOBER 2014. TOPIC: MEASUREMENT AND CHARACTERIZATION OF NANOMATERIALS

### Reactivity and biodegradability of nanomaterials - New end-points for grouping and risk assessment?

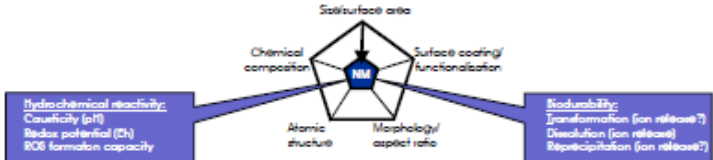
KA Jensen, Y Kembouche, SH Nielsen, and KI Kling  
National Research Centre for the Working Environment, Copenhagen, DENMARK

#### Motivation

There is a need to identify new hazard indicators and robust test methods to enable more reliable grouping principles for read-across and risk assessment of manufactured nanomaterials (NM). Here we present two methods to determine the hydrochemical reactivity and biodegradability (solubility) of NM in different synthetic biological fluids and cell-media used in toxicological testing. The procedures may be applicable for future testing and hazard grouping of particulate matter in general.

#### Challenges in physicochemical grouping of NM

Chemicals are typically grouped based on their chemical identity and primary physicochemical characteristics. However, the toxicity of manufactured materials and in particular to NM may differ from that of their nearest analogue material. This is due to as yet-unknown effects and biological properties, as well as tailored chemical compositions, dopants, surface coatings and functionalizations, atomic structure, morphology and chemical reactivity.



#### Hydrochemical reactivity:

- Causality (pH)
- Redox potential (ORP)
- ROS formation capacity

#### Biodegradability:

- Transformation (on release?)
- Dissolution (on release?)
- Reprecipitation (on release?)

#### The Sensor Dish Reader Method (SDR)

The SDR method was developed to test the hydrochemical reactivity and NM dissolution under in vivo test conditions using a commercially available pH and O<sub>2</sub> Sensor Dish Reader (SDR) system available from BioSens (BioSens - Reaction Sensors GmbH, Bielefeld, Germany). The

#### The Atmosphere-Temperature-pH-controlled Stirred Batch Reactor Method (ATemPH SBR)

The ATemPH SDR method was developed to enable highly controlled analysis of the hydrochemical reactivity and NM dissolution using a

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

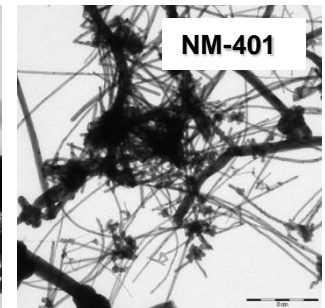
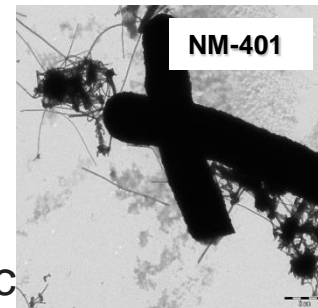
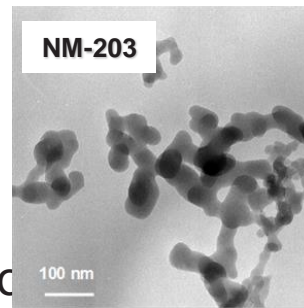
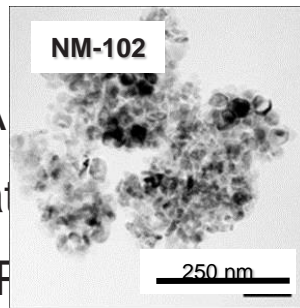
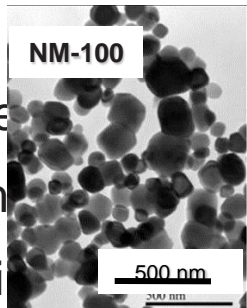
- **Number size-distribution**

- 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

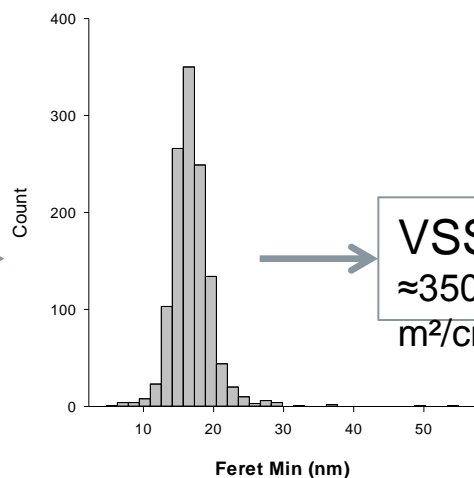
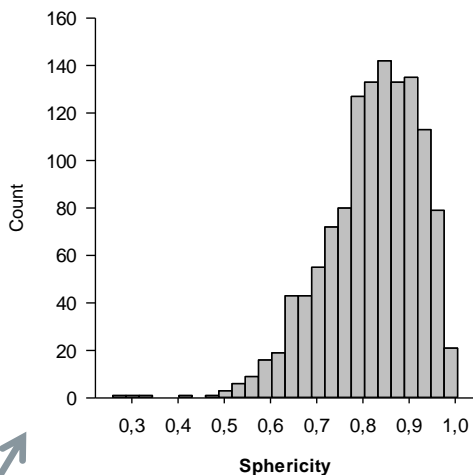
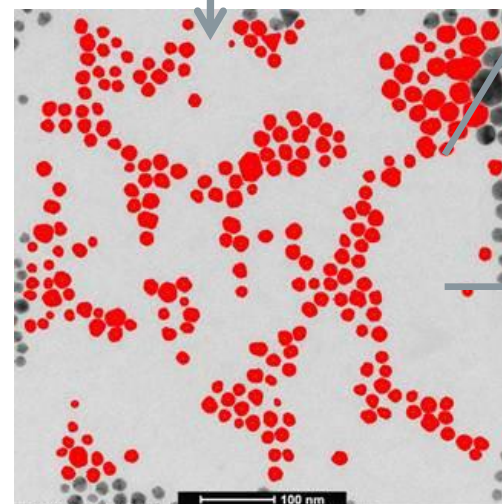
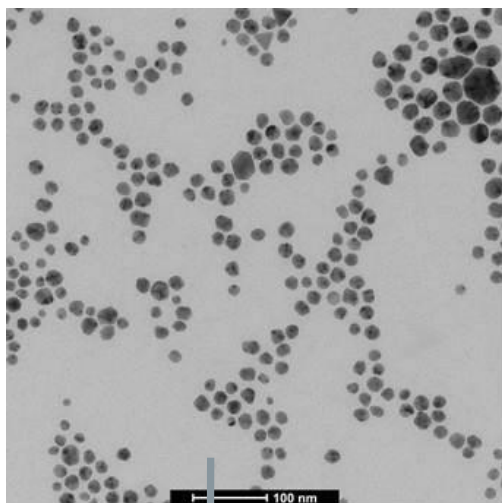
- Proof of principle: identification of primary particles in aggregates/agglomerates

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



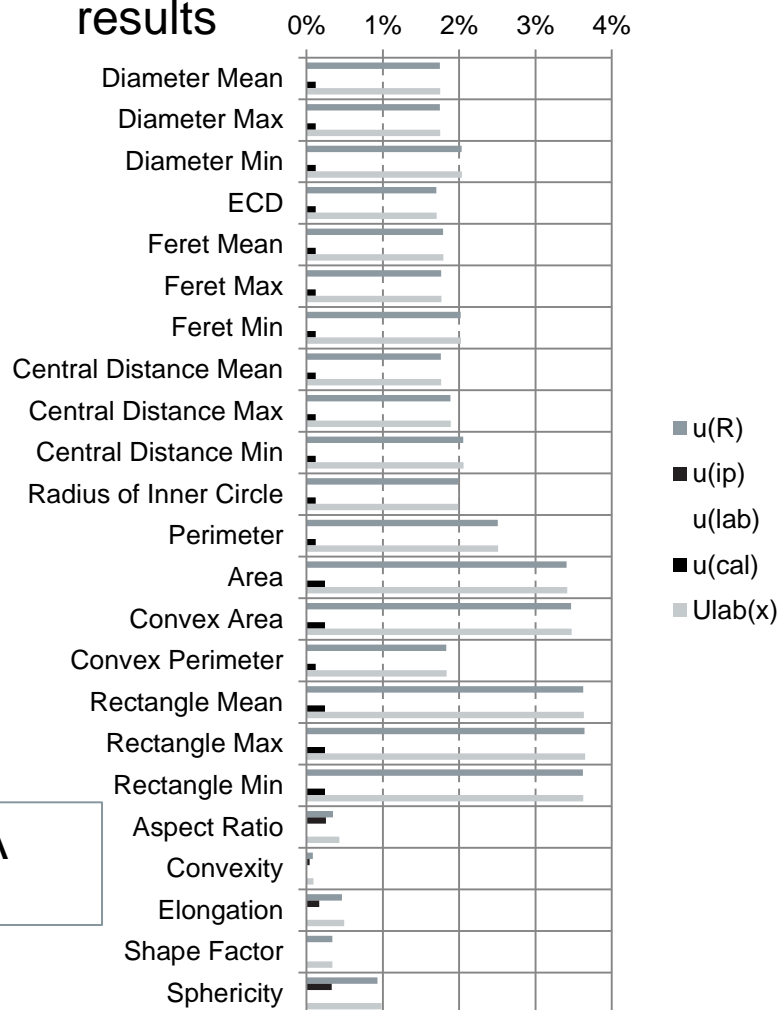
# TEM characterisation of NM

Semi-automated measurement of physical particle properties



VSSA  
 $\approx 350$   
 $m^2/cm^3$

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<sub>2</sub> (NM-10x)
  - ZnO (NM-11x)
  - SAS (NM-20x)
  - CeO (NM-21x)

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

**Derivation: > 20 size parameters and VSSA**

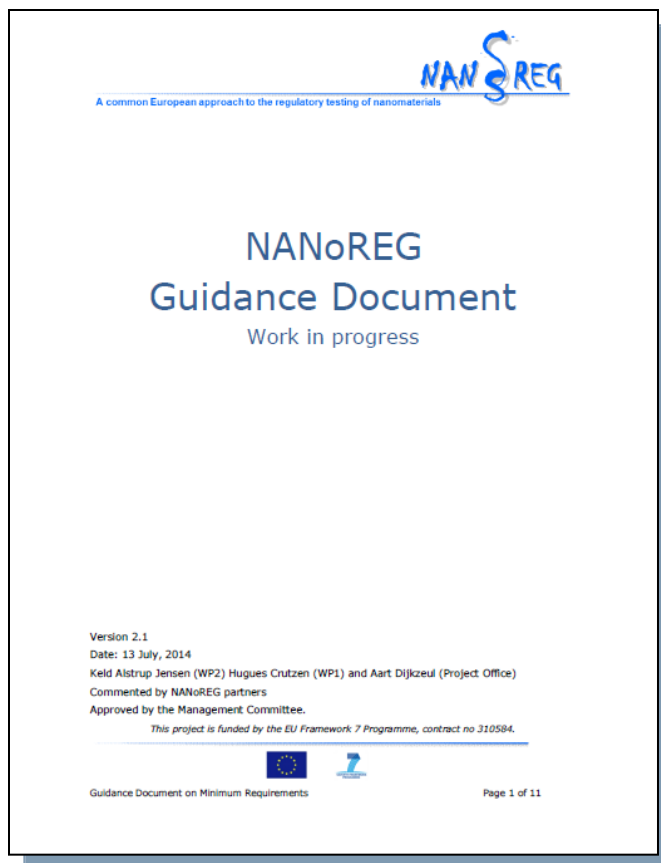
## Pending outcomes

- NANoREG
  - Colloidal
  - Mixtures and nanorods
  - Aggregates and complex NM
- NanoDefine
  - CaCO<sub>3</sub>
  - Pigment yellow
  - BaSO<sub>4</sub>
  - Difficulties expected for
    - Nanoplates like nanosteel
    - Kaolin

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

# Task 2.4: Test item preparation, 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 preparation, qualitative TEM analysis
- ✓ Reporting requirements to NANoREG data-base





# Aims of the Technical Guidance Document

Probe-sonicator calibration protocol

Harmonize de-agglomeration 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	Calorimetric 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	Calorimetric 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)
<b>Nanomaterial check</b>	(R)
<b>Batch dispersion</b>	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).
<b>Initial exposure medium</b>	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)
<b>Final exposure medium</b>	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).
<b>Stability of dispersion during assay</b>	(R)
<b>Contextual conditions and reactivity in the during testing</b>	Measure several of the following parameters (pH, T, conductivity, redox potential and the CO <sub>2</sub> /O <sub>2</sub> concentrations) during testing. In vitro (R) and eco-toxicity (M).
<b>Dissolution in batch dispersion and test media<sup>€</sup></b>	(R)

# Why DLS as the common tool?

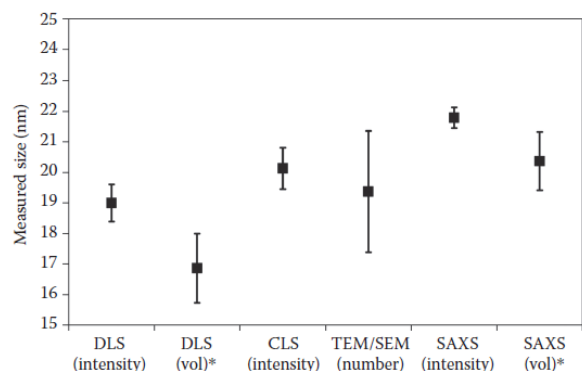
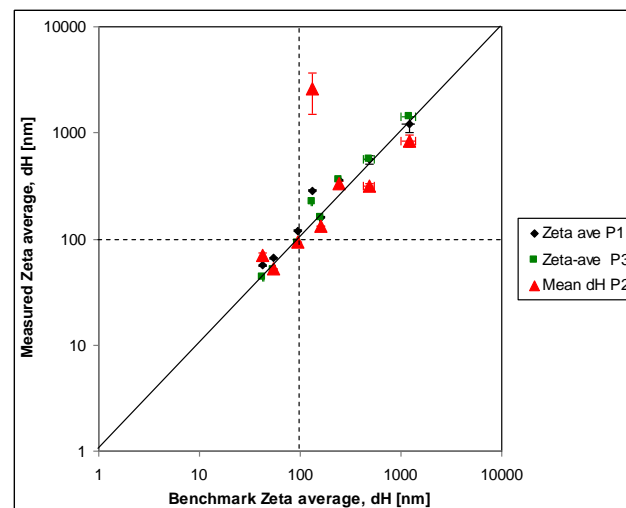


FIGURE 4.2 Size measurement data from the establishment of the certified reference material ERM-FD100 (spherical colloidal silica) using different measurement techniques and units applicable for the specific techniques: standard deviations include measurement uncertainty and interlaboratory variability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

Less user-dependency and highly sensitivity 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 comparability between analysis of dispersions in different laboratories



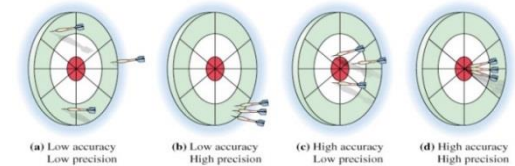
**Is this type of guidance characterization and harmonization and really needed?**

**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)



**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 shortest 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-platey 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 platey 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  $60 \text{ m}^2/\text{cm}^3$  is a spherical equivalent to 100 nm.
  - Limit is a political decision
  - The procedure appears generally not to be too overprotective because 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 pycnometry 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.