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

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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
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

- Accelerating regulatory process
- Scientific answers to regulatory issues
- Credibility regulatory context
- Keeping pace with innovation
NANoREG’s Organisational Structure

General Assembly
(Chair Coordinator)

Global (EU-US)
Community of Research

DG Research and
Innovation

Tom van Teunenbroek
Coordinator

Management
Committee
(Chair Coordinator)

Industry
Consultation Committee

Scientific and
Regulatory Advisory

National Advisory Board

IPR
Advisory Committee

WP1
Scientific answers to regulatory issues

WP2
Synthesis, supplying and characterization

WP3
Exposure through life cycle analysis

WP4
Biokinetics and toxicity testing in vivo

WP5
Regulatory risk assessment and testing

WP6
Keeping pace with Innovation

WP7
Liaisons, dissemination, exploitation, communication

WP8
Project Management

Working Group 1: Solution strategies answering regulatory demands

Working Group 2 Carbon Nanotubes

Value Chain Projects defined by WP 1
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

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

![Graph showing 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 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
Task 2.2: Identification of MNM according to the EC regulatory definition

- **Number size-distribution**
- TEM
- Particle Tracking Analysis
- Dynamic Light Scattering
- Scanning Mobility Particle Sizer coupled with electrospray

- **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

Validation of measurement results

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<thead>
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<th>0%</th>
<th>1%</th>
<th>2%</th>
<th>3%</th>
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<td>Diameter Max</td>
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<td>Diameter Min</td>
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<td>Feret Min</td>
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<td>Central Distance Mean</td>
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<td>Central Distance Max</td>
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<td>Central Distance Min</td>
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<td>Radius of Inner Circle</td>
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<td>Perimeter</td>
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<td>Area</td>
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<td>Convex Area</td>
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<td>Convex Perimeter</td>
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<td>Rectangle Max</td>
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<td>Rectangle Min</td>
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<td>Aspect Ratio</td>
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<td>Convexity</td>
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<td>Elongation</td>
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<td>Shape Factor</td>
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<td>Sphericity</td>
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</table>

VSSA $\approx 350 \text{ m}^2/\text{cm}^3$
Status of semi-automated TEM method:

**Verified**
- Colloidal
  - Gold NIST RM
  - SAS silica CRM
  - Ag (NM-30x)
  - Au (NM-33x)
- Powdered
  - TiO₂ (NM-10x)
  - ZnO (NM-11x)
  - SAS (NM-20x)
  - CeO (NM-21x)

**Pending outcomes**
- NANoREG
  - Colloidal
  - Mixtures and nanorods
  - Aggregates and complex NM
- NanoDefine
  - CaCO₃
  - Pigment yellow
  - BaSO₄
  - 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

**Source:** De Temmerman, E Verleisen, J Mast (CODA CERVA)
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)
- Exposure characterization methods and protocols
- Harmonized reporting ease check of comparability between tests
- Interpretation, interpolation, extrapolation, read-across ....
## Dispersion protocols

<table>
<thead>
<tr>
<th>Type of test</th>
<th>Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibration of sonicators for in vitro and in vivo studies</td>
<td>Calorimetric method combined with adjustment using the NM200 benchmark material NANOGENOTOX batch medium</td>
</tr>
<tr>
<td>In vitro studies</td>
<td>NANOGENOTOX</td>
</tr>
<tr>
<td>In vivo studies</td>
<td>NANOGENOTOX or ENPRA</td>
</tr>
<tr>
<td>Calibration of sonicators for ecotox studies</td>
<td>Calorimetric method combined with adjustment using the NM200 benchmark material in water</td>
</tr>
<tr>
<td>Eco-toxicity studies</td>
<td>A NANoREG water and a NOM*-water protocol for CNT</td>
</tr>
</tbody>
</table>

* Natural Organic Matter

- **Probe-sonicator calibration protocol developed in collaboration with NANODEFIN 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

<table>
<thead>
<tr>
<th>Element in the workflow</th>
<th>Recommendation (R) and Mandatory requirement (M); Optional (O)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nanomaterial check</strong></td>
<td>(R)</td>
</tr>
<tr>
<td><strong>Batch dispersion</strong></td>
<td>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).</td>
</tr>
<tr>
<td><strong>Initial exposure medium</strong></td>
<td>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).</td>
</tr>
<tr>
<td><strong>Final exposure medium</strong></td>
<td>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).</td>
</tr>
<tr>
<td><strong>Stability of dispersion during assay</strong></td>
<td>(R)</td>
</tr>
<tr>
<td><strong>Contextual conditions and reactivity in the during testing</strong></td>
<td>Measure several of the following parameters (pH, T, conductivity, redox potential and the CO₂/O₂ concentrations) during testing. In vitro (R) and eco-toxicity (M).</td>
</tr>
<tr>
<td><strong>Dissolution in batch dispersion and test media</strong></td>
<td>(R)</td>
</tr>
</tbody>
</table>
Why DLS as the common tool?

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-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 m²/cm³ 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.