Deliverable Report for Grant Agreement Number 760813

Deliverable 1.1

Materials acquisition, physicochemical intrinsic properties and endotoxin evaluation on Tier 1 and 2 ENM

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Lead beneficiary for this deliverable: *BASF*

Dissemination Level:		
PU	Public	
PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including the Commission Services)	
CO	Confidential, only for members of the consortium (including the Commission Services)	X

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1. Description of task

Task 1.1 ENM acquisition, collecting intrinsic properties for Tier 1 and 2 ENM and endotoxin evaluation; (**BASF**, ISTEC, NRCWE, KRISS, AMI); **M1-18.**

Materials with high regulatory and industrial relevance have been identified from the French inventory (2016). Tier 1 OECD ENM subsampled or newly synthesised material sub-samples will be accessible from JRC-Ispra and the Fraunhofer Institute or in some cases commercially available (Table 1). Relevant NanoDefine materials will be sourced from the NanoDefine archive at JRC-Geel (also as sub-sampled and characterised ENM). All sample ordering will be managed through the nanomaterial web-order system established as part of the EU FP7 NANoREG, which directly links “customers” and different material providers for easy order management. For Tier 1 materials, the minimum set of information useful for their identification (size- and shape-distribution, surface area/porosity, spectral data on structure and quantitative chemical composition including impurities and surface chemistry and potential presence of chemical doping) will be collected and gaps filled by all task partners. This information will be shared with the consortium during the first month of the project. Tier 2 materials, representing physicochemical (PC) design variations of the Tier 1 selection, will be collected up to M18. These materials will be applied to challenge the new *in vitro* and *in silico* models developed and test their ability to predict hazards of structural analogues, experimentally justify grouping hypotheses and promote safety by design approaches. Special attention will be paid to the potential presence of endotoxin contamination, which will be assessed by AMI using e.g. the Limulus Amebocyte Lysate test. In addition to target materials this task will also collect and distribute to Task 1.2 the specific biological media used for all *in vitro* mammalian and ecological test systems (as prioritised by partners from **WP3-5**) to mimic the acellular environment(s) for ENM characterisation. Technical data sheets will be provided and updated monthly for direct access via the nanomaterial web-order system established by the NRCWE during the NANoREG project and entries will be exportable to the PATROLS database in **WP6**.

Within the PATROLS PC strategy, the present deliverable reports on ‘intrinsic properties’ (what they are).

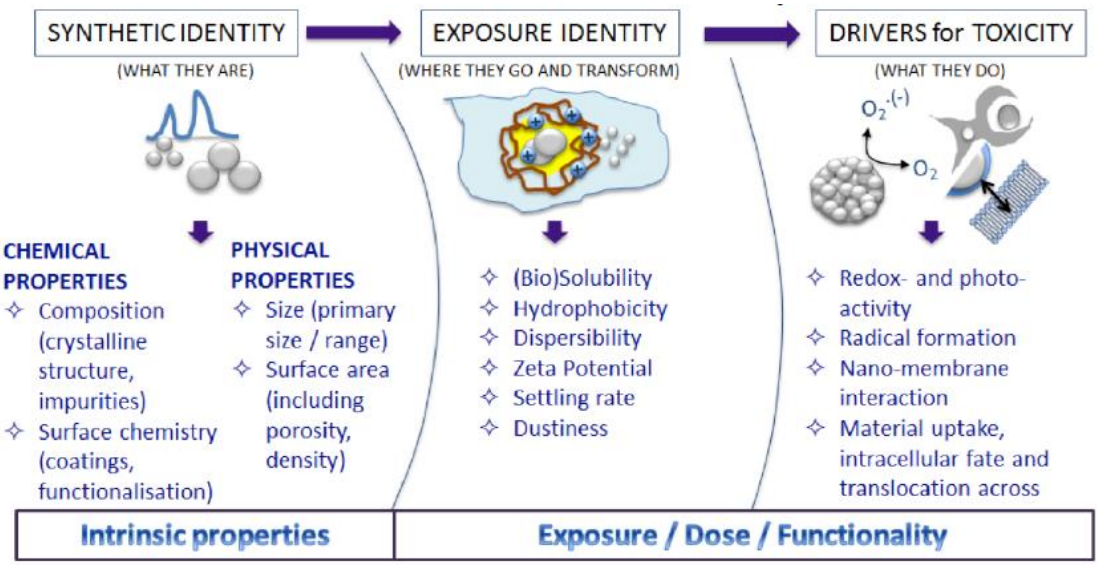

Fig. 1. PATROLS testing strategy for nanomaterial PC characterization and dosimetry.

2. Description of work & main achievements

BASF was responsible for coordinating the collection, generation and curation of physico-chemical characterization of pristine ENM (Tier 1 and 2).

Materials with high regulatory and industrial relevance have been identified from the French inventory "R-nano", which is based on mandatory industrial reporting of all materials produced or imported in nanoform (Ministère de l'Environnement 2015). Tier 1 PATROLS materials were selected from OECD ENM, were subsampled or newly synthesised material sub-samples were made accessible from JRC-Ispra and the Fraunhofer Institute via the PATROLS web-order system (Table 1). A guidance document described in detail how to order materials: "Test Materials and Guidance on the use of the PATROLS Material Information and Web-Order Tool Version 1.0", Steering Board submission date: 27/04/2018 (NRCWE).

For Tier 1 materials, the information on physicochemical intrinsic properties that existed from previous projects was made available to the consortium via excel tables accessible to all PATROLS partners via the WP1 PATROLS server. The information was available from the first month of the project, and was regularly updated.

Additionally, a structured decision (documented in Annex) lead to the selection of Tier 2 materials: (a) MWCNT NM400, (b) TiO_2 E171, (c) ZnO NM113 and (d) SiO_2 NM200. This selection was made during meetings and teleconferences in accordance with the criteria defined in the DoA. The rationale behind this decision was to be able to compare the different physicochemical properties of TIER 1 vs TIER 2 materials. In this way, it was possible to consider: (a) rigid vs flexible MWCNT, (b,c) nano vs non-nano particles, (d) different NM dispersibility and high vs low solubility. The tier 2 materials, representing PC variations of the Tier 1 selection, were collected up to M15, then characterised. Two of the Tier 2 materials complement a nanoform (Tier 1) with a non-nanoform (Tier 2) of the same substance, and thus test the ability of PATROLS methods to predict hazards of structural analogues, to experimentally justify read-across hypotheses. Another two Tier 2 materials complement a nanoform (Tier 1) with another nanoform (Tier 2) of the same substance, and thus test the ability of PATROLS methods to predict hazards of structural analogues.

The minimum set of information acquired were: composition (X-ray photoelectron spectroscopy, XPS, and X-ray fluorescence, XRF), crystallinity (X-ray diffraction, XRD), size (transmission electron microscopy, TEM, and scanning electron microscopy, SEM), coating (thermal gravimetric analysis, TGA), density (He-pycnometer), surface charge (Z-potential), and hydrophobicity (water contact angle). Such characteristics were collected or generated by different partners: BASF (analytical ultracentrifuge, BET, He-pycnometer, contact angle, Z-potential), KRISS (XPS, XRD), ISTEC-CNR (TEM, SEM) and NRCWE (XRF, TGA). The possible presence of endotoxin was detected by AMI using the Limulus Amebocyte Lysate test and generating an SOP (ANNEX III).

Below there is the link to reach PATROLS server, where there is the direct access to NANoREG templates (D1.1 intrinsic physchem data PATROLS_final and PATROLS_NANoREG templates_final). Such template contains the full sample characterization with all method parameters collected in this task. The server address is: https://patrolsproject.webdav.hidrive.strato.com/users/patrolsproject

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We thus ensured that all results are directly importable into the PATROLS database in **WP6**, by documenting results in the pre-defined NANoREG templates that comply with enanomapper requirements and with the NANoREG ontology.

a. Materials: Tier 1

At first tier selection of materials, the PATROLS DoA selected materials that represent the main groups of 1. Soluble ENM, 2. Biopersistent HARN, 3. Passive ENM; 4 active ENM (and Quartz). Theses have been selected due to the documented availability of in vivo chronic / sub-chronic exposure data, useful for the verification of models generated by the project. The list, with repositories where materials are stored, is reported in the Table below, reproduced from the DoA.

Tier 1	ENM & Supplier	Available form
1: Soluble (release possibly toxic ions)	ZnO (NM111; JRC) COATED	Powder
	ZnO (NM110; JRC) UNCOATED	Powder
	Ag (NM300K; Fraunhofer IME)	Suspension (10 wt.%)
	Ag Sigma-576832	Powder
2: Biopersistent HARN (fibre paradigm)	Mitsui-7 MWCNT (NRCWE-006)	Powder
	MWCNT (NM402; JRC)	Powder
3: Passive (no reactive or toxic potential)	$BaSO_4$ (NM220; Fraunhofer IME)	Powder
4: Active (positive, insoluble; promote cellular effects and/or mobility in the organism)	CeO2 (NM212; Fraunhofer IME)	Powder
	Alpha_crystalline SiO_2 > 5mm	Powder
	Amorphous silica	Powder
	TiO_2 (NM105; JRC)	

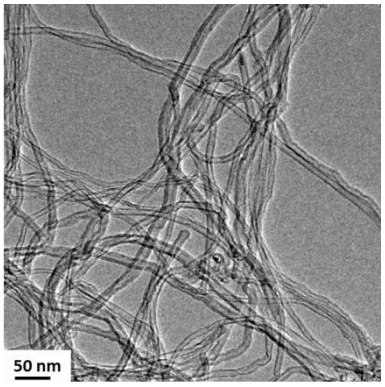
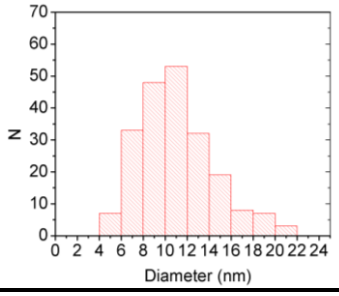
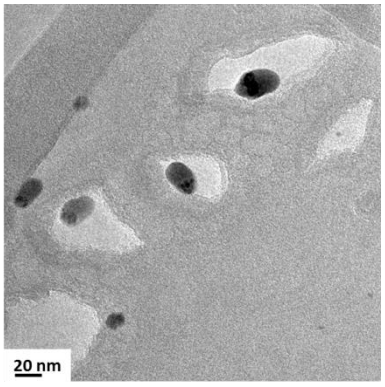
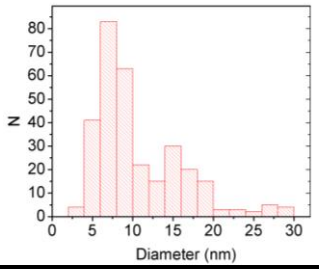
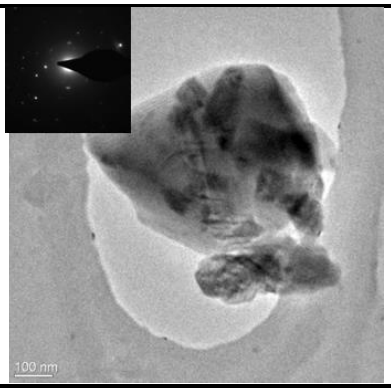
b. Materials: Tier 2

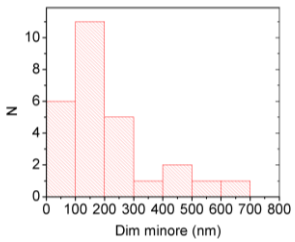
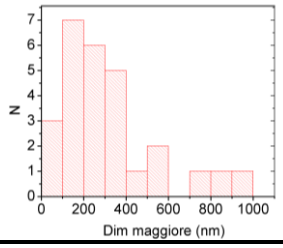
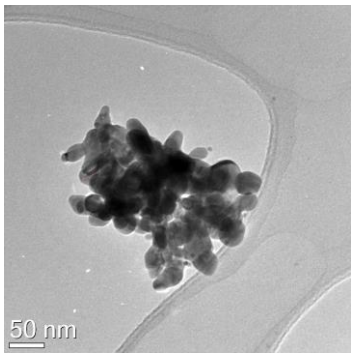
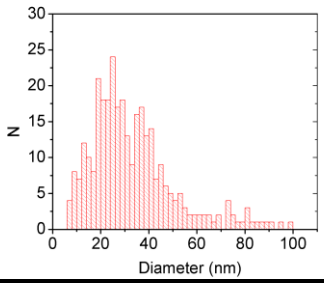
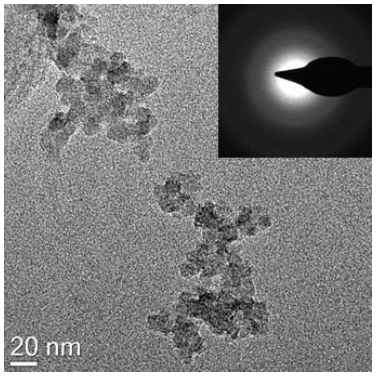
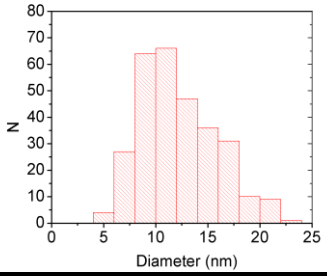
At a second tier, the materials library has been extended in order to cover a wider range of materials and test the capacity of the methods developed in PATROLS for discriminating between different classes of materials. The rationale behind the selection of Tier 2 materials was to be able to compare: rigid vs flexible MWCNT; nano vs non-nano particles ZnO and different synthetic routes for SiO_2 (precipitated vs pyrogenic).

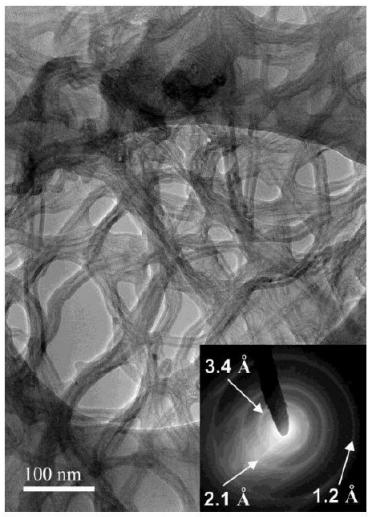
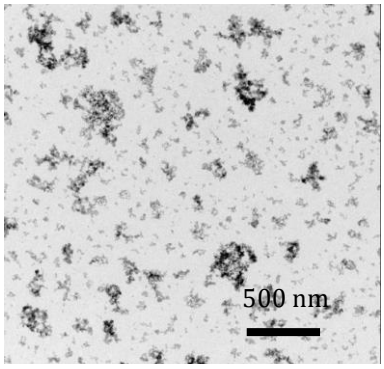
Tier 2	Comparison to Tier 1 material	Available form
TiO_2 E171 (food grade) (non nano)	TiO_2 NM105 (nano)	Powder
SiO_2 NM200 (precipitated)	SiO_2 amorphous (pyrogenic)	Powder
ZnO NM113 (non nano)	ZnO NM 110 (nano)	Powder
MWCNT NM400 (NC7000) (flexible)	MWCNTs Mitsui (rigid)	Powder

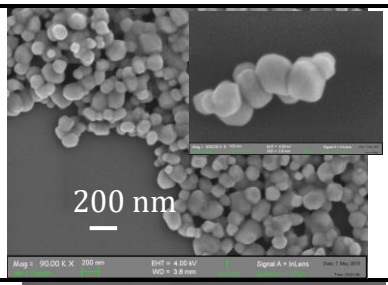
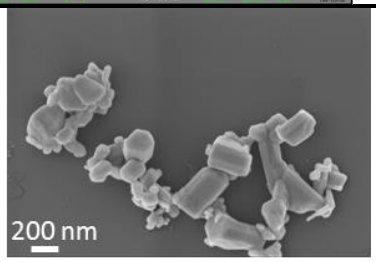
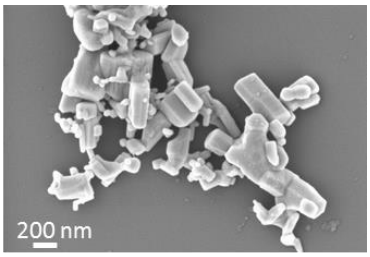
c. Characterisation results of Tier 1 and Tier 2 materials

Below there is the collection of representative electron microscopy pictures of Tier 1 and Tier 2 analyzed by ISTEC.

Tier 1 Samples	Representative TEM Image	Statistical distribution	Comments
CNT NM402		Thicknesses of more than 200 nanotubes in different plates were measured. The estimated average diameter is 10.5 nm with a standard deviation of 3.4 nm. Therefore with a confidence interval of 99% we assumed an average diameter value D_{CNT}=(10.5±0.7) nm. 	Phase contrast TEM images show nanotubes of different diameters. The length of the nanotubes is estimated to be between 0.5 and 2 µm.
Ag NM300		The statistical distribution of the particle size is bimodal: it is possible to identify two groups of particles one with an average diameter of (7.3 ± 0.4) nm and the other with an average diameter of (16 ± 1) nm. The associate errors are calculated for a confidence interval of 99%. 	Phase contrast TEM images clearly show the presence of particles having different sizes. In particular it is possible to detect two different characteristic sizes.
Crystalline SiO_2 DQ12 IOM		Estimated values from the two distributions of size are: $<D_{min}>$ =207 nm St Dev =155 nm $<D_{max}>$ =307 nm St Dev =232 nm. Therefore, with a confidence interval of 99% we assumed an average diameter of D_{min}=(200±30) nm e D_{max}=(300±50) nm.	Bright field TEM images show fragments, the maximum and minimum dimensions were measured. The particles are crystalline as evidenced by the Selected Area Electron Diffractions (SAED).

		 	
Ag SIGMA 576832		The statistical distribution of the particle size is three-modal: it is possible to identify three groups of particles: one with a median of 12 nm, the second distribution exhibits a median of 25 nm and the last one has a mean equal to 43 nm. 	Phase contrast TEM images that clearly show the presence of particles having different size. In particular it is possible to detect three different characteristic sizes.
Amorphous SiO_2 IUF		The statistical sampling consists of 295 particles. The distribution exhibits, with a confidence interval of 99%, an average diameter of the SiO_2 clusters of 11.7 ± 0.7nm 	Phase contrast TEM images showing the presence of SiO_2 particles having a size ranging from 5 to 20 nm. The particles are amorphous as evidenced by the SAED.

Tier 2 Samples	Representative TEM Image	Statistical distribution	Comments
MWCNT NM400		The thicknesses of more than 200 nanotubes in different plates were measured. The estimated mean diameter is D_{CNT}=(10.5±0.7) nm, with a Geodesic length calculated: 846±446nm, and a resulting aspect ratio of 79±50nm.	TEM micrograph and analysis performed by CODA-CERVA laboratory (Belgium) and reported in JRC report 2014 (Multi-walled Carbon Nanotubes, NM-400, NM-401, NM-402, NM-403: Characterization and PC Properties.
SiO_2 NM200		TEM micrograph of NM-200 shows the complex structure of silicon dioxide aggregates. The general morphology of the primary sub-units of NM-200 is equi-axed and rounded, or slightly elongated with a suggested spherical or ellipsoidal 3D structure. The statistical distribution (for 4997 particles) estimated a mean diameter of 50±55nm.	TEM micrograph and analysis performed by CODA-CERVA laboratory (Belgium) and reported in JRC report 2013 (Synthetic Amorphous Silicon Dioxide (NM-200, NM-201, NM-202, NM-203, NM-204): Characterization and PC Properties).

Samples	SEM-FEG Image	Statistical distribution	Comments
TiO_2 E171 (food grade)		The statistical sampling over 2082 particles exhibits a mean diameter of 180±57nm.	Nanoparticles form aggregates of elongated shape. Equi-axed and rounded primary particles are detected together with larger particles with a more irregular shape.
ZnO NM113	 	The statistical sampling over 107 particles exhibits a mean diameter of 210±138nm.	FEG-SEM micrographs show the presence of aggregates formed by primary particles with different shapes, with a prevalence of a rod like shape.

On the following pages, we report numerical results in a series of condensed tables, sorted by substances that combine the matching Tier 1 and Tier 2 materials.

The condensed tables are extracted from the full data contained in the NANoREG template.

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			Amorphous SiO_2 IUF				Crystalline SiO_2 DQ12 IOM				SiO_2 NM200			
property	**preferred methods & descriptor**	**unit**	Method / Technique	value	std. dev.	data source (e.g. institute, project, publication)	Method / Technique	value	std. dev.	data source (e.g. institute, project, publication)	Method / Technique	value	std. dev.	data source (e.g. institute, project, publication)
Composition: CAS	identification by CAS no	n/a	CAS	7631-86-9			CAS	14808-60-7			CAS	7631-86-9		
XRD: composition, sum formula	identification by sum formula	n/a	XRD	SiO2		KRISS	XRD	SiO_2		BASF, J. Ceramic. Sci. Tech., 04, 93-104 (2013)	XRD	SiO_2		JRC report
XRD: composition, crystallinity	identification by crystallinity	n/a	XRD	amorphous SiO_2		KRISS	XRD	Quartz, SiO_2 hexagonal		BASF, J. Ceramic. Sci. Tech., 04, 93-104 (2013)	XRD	amorphous SiO_2		Rasmussen et al. 2013 JRC report
XRF: impurities	content for each identified impurity <0.1%, <1%, exact value if >1%.	%	XRF	<0.1% (CaO, CuO)		NRCWE	XRF	<1% (CaO, Al2O3); <0.1% (Fe_2O_3, Cr_2O_3, TiO_2, K2O, Na_2O, MgO)		Clouter A et al., Toxicol Sci. 2001, 63(1):90	XRF	>1% (SO_3 2.62%, Na_2O 1.44%); <1% (Cl, Al_2O_3); <0.1% (CaO, TiO_2, Fe_2O_3, K_2O, CuO, NiO, ZrO_2, ZnO)		NRCWE
TGA/DTG: functional groups	Weight loss	%	TGA/DTG	11.5 (confirmative test needed)		NRCWE	TGA/DTG	-2.71		NRCWE	TGA/DTG	-3.29		NRCWE
TEM/SEM: constituent (primary) particle size	NanoDefine methodology	nm	TEM (software evaluation)	mean MinFeret 8.3	3	ISTEC-CNR	TEM (software evaluation)	mean MinFeret 145.8	158.2	ISTEC-CNR	TEM (manual evaluation)*	Feret (min) mean: 35.5	Feret (min) mean: 38.9	Rasmussen et al. 2013 JRC report
	NanoDefine methodology	3D / 2D / 1D nm	TEM (software evaluation)	3D; mean aspect ratio 1.30; mean circularity 0.24	SD aspect ratio 0.93; SD circularity 0.06	ISTEC-CNR	TEM (software evaluation)	3D; mean aspect ratio 1.70; mean circularity 0.81	SD aspect ratio 0.44; SD circularity 0.09	ISTEC-CNR	TEM	Mean aspect ratio: 1.57; Mean circularity: 0.41	Mean aspect ratio: 0.35; Mean circularity: 0.20	Rasmussen et al. 2013 JRC report
Chemical Nature of the Surface: coatings	identification of surface treatment agents	n/a	(descriptive)	none			(descriptive)				(descriptive)			
XPS: chemical nature of the surface	elemental composition of outermost 1nm	atom%	XPS	Si 30.8 %, O 69.2 %		KRISS	XPS	Si 26.5 %, C 10.6 %, O 62.9 %		BASF	XPS	C 4.1%, O 70.8%, Si 24.1%, S 0.06%, Na 1.0%		Rasmussen et al. 2013 JRC report
BET: specific surface area / porosity	specific area	m^2/g	BET	192.92		BASF	BET	11.12		BASF	BET	166.48		BASF
He pycnometer: density	density (skeletal)	g/cm^3	He pycnometry	3.9		BASF	He pycnometry	2.61		BASF	He pycnometry	2.19		BASF
Surface charge	IEP		IEP	3.5		BASF	IEP	< 3		BASF	IEP	3.3		BASF
Surface charge	zeta-potential pH7	mV	zeta-potential at pH7 in 10 mmol/l KCl water	-35		BASF	zeta-potential at pH7 in 10 mmol/l KCl water	-39		BASF	zeta-potential at pH7 in 10 mmol/l KCl water	-22		BASF
AUC: size	D50 number metric (nm)	nm	D50 number metric	52		BASF	D50 number metric	338		BASF	D50 number metric	582		BASF
Water contact angle: surface hydrophobicity	Water contact angle	°	sessile drop: MilliQ water	77.1	1.4	BASF	sessile drop: MilliQ water			BASF	sessile drop: MilliQ water	<10		ECETOC

			CeO_2 NM212				$BaSO_4$ NM220				$BaSO_4$ JRCNM50001a (=same-grade-later-batch, used for NANoREG longterm inhalation)			
Property	**preferred methods & descriptor**	**unit**	Method / Technique	value	std. dev.	data source (e.g. institute, project, publication)	Method / Technique	value	std. dev.	data source (e.g. institute, project, publication)	Method / Technique	value	std. dev.	data source (e.g. institute, project, publication)
Composition: CAS	identification by CAS no	n/a	CAS	1306-38-3			CAS	7727-43-7			CAS	7727-43-7		
XRD: composition, sum formula	identification by sum formula	n/a	XRD	CeO_2		BASF	XRD	$BaSO_4$		BASF	XRD	$BaSO_4$		Konduru et al. Part Fibre Toxicol. 11(1). 55 2014.
XRD: composition, crystallinity	identification by crystallinity	n/a	XRD	cerianite, cubic		BASF	XRD	crystalline, orthorombic		BASF	XRD	crystalline, orthorombic		
XRF: impurities	content for each identified impurity <0.1%, <1%, exact value if >1%.	%	XRF	<1% (P_2O_5, CaO, Cl); <0.1% (V_2O_5, SO_3, CoO, Fe_2O_3, MgO, CuO, SiO_2, ZnO)		NRCWE	XRF	purity > 93.8%, Na, Ca, Sr, F, Cl, organic contaminations		Konduru et al. Part Fibre Toxicol. 11(1). 55 2014.	XRF	purity > 95%		Konduru et al. Part Fibre Toxicol. 11(1). 55 2014.
TGA/DTG: functional groups	Weight loss	%	TGA/DTG	-0.95		NRCWE	TGA/DTG	-4.1		NRCWE	TGA/DTG	not measurable nor relevant		NRCWE
TEM/SEM: constituent (primary) particle size	NanoDefine methodology	nm	TEM (software evaluation)	mean MinFeret 13.7	7.6	BASF	TEM (software evaluation)	mean MinFeret 31.5	15.9	BASF	TEM (manual evaluation)	25	10	Konduru et al. Part Fibre Toxicol. 11(1). 55 2014.
	NanoDefine methodology	3D / 2D / 1D nm	TEM (software evaluation)	3D; mean aspect ratio 1.21; mean circularity 0.97	SD aspect ratio 0.25; SD circularity 0.06	BASF	TEM (software evaluation)	3D; mean aspect ratio 1.22; mean circularity 0.98	SD aspect ratio 0.19; SD circularity 0.04	BASF	TEM	3D		Konduru et al. Part Fibre Toxicol. 11(1). 55 2014.
Chemical Nature of the Surface: coatings	identification of surface treatment agents	n/a	(descriptive)	none		Molina et al. Environ Sci Nano 1561-73. 2014.; Keller et al. Arch. Toxicol. 88 2033-59. 2014.	(descriptive)	none			(descriptive)	none		
XPS: chemical nature of the surface	elemental composition of outermost 1nm	atom%	XPS	Ce 25.6 %, O 74.4 %		KRISS	XPS	Ba 21.5 %, S 12.5 %, O 65.8 %		KRISS	XPS	O 64%, Ba 15%, C 2%, S 17%, Na 2%		Konduru et al. Part Fibre Toxicol. 11(1). 55 2014.
BET: specific surface area / porosity	specific area	m^2/g	BET	27		BASF	BET	33		BASF	BET	38		Konduru et al. Part Fibre Toxicol. 11(1). 55 2014.
He pycnometer: density	density (skeletal)	g/cm^3	He pycnometry	7.2		BASF	He pycnometry	4.13		BASF	He pycnometry	not measurable nor relevant		BASF
Surface charge	IEP		IEP				IEP				IEP			
Surface charge	zeta-potential pH7	mV	zeta-potential at pH7 in xxx water	35.2		BASF	zeta-potential at pH7 in xxx water	-30.2	2	Konduru et al. Part Fibre Toxicol. 11(1). 55 2014.	zeta-potential at pH7 in xxx water	-32	2	Konduru et al. Part Fibre Toxicol. 11(1). 55 2014.
AUC: size	D50 number metric (nm)	nm	D50 number metric	not measurable nor relevant		BASF	D50 number metric	32		W Wohlleben NanoImpact 12 (2018) 29–41	D50 number metric	not measurable nor relevant		BASF
Water contact angle: surface hydrophobicity	Water contact angle	°	sessile drop: MilliQ water	60°	2.1	nanoGRAVUR, BASF	sessile drop: MilliQ water	<10°		nanoGRAVUR, BASF	sessile drop: MilliQ water	not measurable nor relevant		BASF

			ZnO NM111				ZnO NM113			
property	**preferred methods & descriptor**	**unit**	Method / Technique	value	std. dev.	data source (e.g. institute, project, publication)	Method / Technique	value	std. dev.	data source (e.g. institute, project, publication)
Composition: CAS	identification by CAS no	n/a	CAS	1314-13-2			CAS	1314-13-2		
XRD: composition, sum formula	identification by sum formula	n/a	XRD	ZnO		KRISS	XRD	ZnO		KRISS
XRD: composition, crystallinity	identification by crystallinity	n/a	XRD	ZnO (Zincite)		KRISS	XRD	(Zincite)		KRISS
XRF: impurities	content for each identified impurity <0.1%, <1%, exact value if >1%.	%	XRF	>1% (Na_2O 1.1%); <0.1% (P_2O_5, SiO_2, CaO, CuO, Fe_2O_3, NiO)		NRCWE	XRF	<0.1% (NiO, Fe_2O_3, CuO)		NRCWE
TGA/DTG: functional groups	Weight loss	%	TGA/DTG	-2.67		NRCWE	TGA/DTG	-1.06		NRCWE
TEM/SEM: constituent (primary) particle size	NanoDefine methodology	nm	TEM (software evaluation)	mean MinFeret 40.6	26.1	ISTEC-CNR	TEM (software evaluation)	mean MinFeret 166.9	116.8	ISTEC-CNR
	NanoDefine methodology	3D / 2D / 1D nm	TEM (software evaluation)	3D; mean aspect ratio 1.88; mean circularity 0.80	SD aspect ratio 0.78; SD circularity 0.12	ISTEC-CNR	TEM (software evaluation)	3D; mean aspect ratio 1.71; mean circularity 0.83	SD aspect ratio 0.80; SD circularity 0.12	ISTEC-CNR
Chemical Nature of the Surface: coatings	identification of surface treatment agents	n/a	(descriptive)	UV-active silicon coating triethoxycaprylsilane		Landsiedel et al. Part Fibre Toxicol. 11(1). 16. 2014	(descriptive)			
XPS: chemical nature of the surface	elemental composition of outermost 1nm	atom%	XPS	Zn 34.6 %, C 22.2 %, O 43.1 %		KRISS	XPS	Zn 49.63 %, C 6.92 %, O 43.45 %		KRISS
BET: specific surface area / porosity	specific area	m²/g	BET	12		BASF	BET	11.01		BASF
He pycnometer: density	density (skeletal)	g/cm³	He pycnometry	4.99		BASF	He pycnometry	5.49		BASF
Surface charge	IEP		IEP				IEP	9.7		BASF
Surface charge	zeta-potential pH7	mV		not measurable nor relevant			zeta-potential at pH7 in 10 mmol/l KCl water	32*	* sample dissolves upon the addition of acid	BASF
AUC: size	D50 number metric (nm)	nm	D50 number metric	not measurable nor relevant		BASF	D50 number metric	not measurable nor relevant		BASF
Water contact angle: surface hydrophobicity	Water contact angle	°	sessile drop: MilliQ water	152°	4.4	nanoGRAVUR, BASF	sessile drop: MilliQ water	126°	4	BASF

			Ag Sigma 576832				Ag NM300			
property	**preferred methods & descriptor**	**unit**	Method / Technique	value	std. dev.	data source (e.g. institute, project, publication)	Method / Technique	value	std. dev.	data source (e.g. institute, project, publication)
Composition: CAS	identification by CAS no	n/a	CAS	7440-22-4			CAS	7440-22-4		
XRD: composition, sum formula	identification by sum formula	n/a	XRD	Ag		KRISS	XRD	Ag + amorphous (91.4% : 8.6%). Ag2O + Ag (93% : 7%)		KRISS
XRD: composition, crystallinity	identification by crystallinity	n/a	XRD	Ag		KRISS	XRD			KRISS
XRF: impurities	content for each identified impurity <0.1%, <1%, exact value if >1%.	%	XRF	<1% (Pd, Cl); <0.1% (Rh, Fe, Cu, Ni)		NRCWE	XRF	<1% (CaO, P_2O_5, Pd); <0.1% (Cl, CdO, K_2O, Fe_2O_3, SiO_2, CuO, NiO, MoO_3)		NRCWE
TGA/DTG: functional groups	Weight loss	%	TGA/DTG	-3.37		NRCWE	TGA/DTG	- 83.63 (NANoREG D 2.4); - 72.26 (NFA SOP)		NRCWE
TEM/SEM: constituent (primary) particle size	NanoDefine methodology	nm	TEM (software evaluation)	mean MinFeret 30.0	23.9	ISTEC-CNR	TEM (software evaluation)	mean MinFeret 7.2	4.3	ISTEC-CNR
	NanoDefine methodology	3D / 2D / 1D nm	TEM (software evaluation)	3D; mean aspect ratio 1.36; mean circularity 0.88	SD aspect ratio 0.30; SD circularity 0.09	ISTEC-CNR	TEM (software evaluation)	3D; mean aspect ratio 1.20; mean circularity 0.98	SD aspect ratio 0.23; SD circularity 0.04	ISTEC-CNR
Chemical Nature of the Surface: coatings	identification of surface treatment agents	n/a	(descriptive)				(descriptive)	Identified two peaks (bimodal distribution)		
XPS: chemical nature of the surface	elemental composition of outermost 1nm	atom%	XPS	Ag 38.9 %, C 47.6%, O 13.5 %		KRISS	XPS	Ag 1.4 %, C 71.3 %, O 27.2 %		KRISS
BET: specific surface area / porosity	specific area	m^2/g	BET	6.43		BASF	BET	not relevant (suspension)		BASF
He pycnometer: density	density (skeletal)	g/cm^3	He pycnometry	8.36		BASF	He pycnometry	not relevant (suspension)		BASF
Surface charge	IEP		IEP	< 3		BASF	IEP	3.9		BASF
Surface charge	zeta-potential pH7	mV	zeta-potential at pH7 in 10 mmol/l KCl water	-30		BASF	zeta-potential at pH7 in 10 mmol/l KCl water	-22		BASF
AUC: size	D50 number metric (nm)	nm	D50 number metric	90		BASF	D50 number metric	12		BASF
Water contact angle: surface hydrophobicity	Water contact angle	°	sessile drop: MilliQ water	140.8	1	BASF	sessile drop: MilliQ water	not measurable nor relevant		BASF

			MWCNT NM402				MWCNT NM400				Mitsui NT7 (NRCWE_006)			
property	**preferred methods & descriptor**	**unit**	Method / Technique	value	std. dev.	data source (e.g. institute, project, publication)	Method / Technique	value	std. dev.	data source (e.g. institute, project, publication)	Method / Technique	value	std. dev.	data source (e.g. institute, project, publication)
Composition: CAS	identification by CAS no	n/a	CAS	308068-56-6			CAS	308068-56-6			CAS	308068-56-6		
XRD: composition, sum formula	identification by sum formula	n/a	XRD	MWNT with small amount of impurities (amorphous carbon, debri, catalyst and so on)		KRISS	XRD	MWNT with small amount of impurities (amorphous carbon, debri, catalyst and so on)		KRISS	XRD	MWCNT (Fe_2O_3 observed in sample after TGA)		NRCWE NANOGENOTOX D4.1
XRD: composition, crystallinity	identification by crystallinity	n/a	XRD			KRISS	XRD			KRISS	XRD			
XRF: impurities	content for each identified impurity <0.1%, <1%, exact value if >1%.	%	XRF	2.43% Al_2O_3, 1.31% Fe_2O_3; <0.1% CaO, CuO, MgO, NiO, P_2O_5, ZnO)		NRCWE (Jackson et al., Environmental and MolecularMutagenesis 56:183-203 (2015)	XRF	>1% (Al 2.894 %), <1% (Fe); <0.1% (Co, Na, Cr, Ni, Zn, Cu)		NRCWE	XRF	0.14% P_2O_5; <0.1% (CuO, Fe_2O_3, MgO, SiO_2, SO_3, ZnO)		NRCWE (Jackson et al. Environmental and MolecularMutagenesis 56:183-203 (2015)
TGA/DTG: functional groups	Weight loss	%	TGA/DTG	-90.93		NRCWE	TGA/DTG	-90.46		NRCWE	TGA/DTG	-98.71		NRCWE
TEM/SEM: constituent (primary) particle size	NanoDefine methodology	nm	TEM (software evaluation)	diameter: 10.5; 0,5 mm < Lenght < 2mm;	diameter: 3.4	ISTEC-CNR	TEM (software evaluation)	Thickness: 11; Lenght: 846	Thickness: 3; Lenght: 446	Rasmussen et al. 2014 JRC report	TEM (software evaluation)	Diameter: 74 Length: 5730	Diameter: 28 Length 3674	CODA CERVA NANOGENOTOX D4.1
	NanoDefine methodology	3D / 2D / 1D nm	TEM (software evaluation)	Tubes, Mean aspect ratio: >100		ISTEC-CNR	TEM (software evaluation)	aspect ratio: 79	aspect ratio: 50	Rasmussen et al. 2014 JRC report	TEM (software evaluation)	Aspect ratio: 85	Aspect ratio 65	CODA CERVA NANOGENOTOX D4.1
Chemical Nature of the Surface: coatings	identification of surface treatment agents	n/a	(descriptive)	Poly-dispersed distribution of diameter			(descriptive)				(descriptive)			
XPS: chemical nature of the surface	elemental composition of outermost 1nm	atom%	XPS	Pure C (graphite-like), C-O, O-C-O		KRISS	XPS	Pure C (graphite-like)		KRISS	XPS	Pure C (graphite-like) C-O		KRISS
BET: specific surface area / porosity	specific area	m^2/g	BET	240.49		BASF	BET	254		BASF NANOGRAVUR	BET	22		IMC-BAS NANOGENOTOX D4.1
He pycnometer: density	density (skeletal)	g/cm^3	He pycnometry	2.07		BASF	He pycnometry	1.8		BASF	He pycnometry			
Surface charge	IEP		IEP	3.8		BASF	IEP	not measurable nor relevant		BASF	IEP	not measurable nor relevant		
Surface charge	zeta-potential pH7	mV	zeta-potential at pH7 in 10 mmol/l KCl water	-17	3	BASF	zeta-potential at pH7 in 10 mmol/l KCl water	not measurable nor relevant		BASF	zeta-potential at pH7 in 10 mmol/l KCl water	not measurable nor relevant		
AUC: size	D50 number metric (nm)	nm	D50 number metric	956		BASF	D50 number metric	not measurable nor relevant		BASF	D50 number metric	not measurable nor relevant		
Water contact angle: surface hydrophobicity	Water contact angle	°	sessile drop: MilliQ water	71.1	29.7	BASF	sessile drop: MilliQ water	140		BASF, NANOGRAVUR	sessile drop: MilliQ water			

			TiO_2 E171				TiO_2 NM105			
property	**preferred methods & descriptor**	**unit**	Method / Technique	value	std. dev.	data source (e.g. institute, project, publication)	Method / Technique	value	std. dev.	data source (e.g. institute, project, publication)
Composition: CAS	identification by CAS no	n/a	CAS	13463-67-7			CAS	1317-80-2		
XRD: composition, sum formula	identification by sum formula	n/a	XRD	TiO2		KRISS	XRD	TiO2		KRISS
XRD: composition, crystallinity	identification by crystallinity	n/a	XRD	Anatase		KRISS	XRD	Anatase + Rutile (86.9% : 13.1%)		KRISS
XRF: impurities	content for each identified impurity <0.1%, <1%, exact value if >1%.	%	XRF	<1% (k_2O, P_2O_5); <0.1% (Nb_2O_5, ZrO_2, MoO_3)		NRCWE	XRF	purity >99%		Arts et al. Regul Toxicol Pharmacol 76. 234-261. 2016.; Landsiedel et al. Adv. Mater. 22(24). 2601-2627. 2010.
TGA/DTG: functional groups	Weight loss	%	TGA/DTG	-0.97		NRCWE	TGA/DTG	-3.76		NRCWE
TEM/SEM: constituent (primary) particle size	NanoDefine methodology	nm	SEM (software evaluation)	mean MinFeret 152.0	51.2	ISTEC-CNR	TEM (manual evaluation)	25		Arts et al. Regul Toxicol Pharmacol 76. 234-261. 2016.; Landsiedel et al. Adv. Mater. 22(24). 2601-2627. 2010.
	NanoDefine methodology	3D / 2D / 1D nm	SEM (software evaluation)	Mean aspect ratio: 1.28; Mean circularity: 0.97	Mean aspect ratio: 0.23; Mean circularity: 0.04	ISTEC-CNR	TEM	3D		Arts et al. Regul Toxicol Pharmacol 76. 234-261. 2016.; Landsiedel et al. Adv. Mater. 22(24). 2601-2627. 2010.
Chemical Nature of the Surface: coatings	identification of surface treatment agents	n/a	(descriptive)				(descriptive)	none		
XPS: chemical nature of the surface	elemental composition of outermost 1nm	atom%	XPS	Ti 30.0 %, O 66.4 %, K 3.6 %		KRISS	XPS	Ti 24.5 %, O 65.0 %, C 10.5 %		KRISS
BET: specific surface area / porosity	specific area	m^2/g	BET	10.8		BASF	BET	51		Arts et al. Regul Toxicol Pharmacol 76. 234-261. 2016.; Landsiedel et al. Adv. Mater. 22(24). 2601-2627. 2010.
He pycnometer: density	density (skeletal)	g/cm^3	He pycnometry	3.85		BASF	He pycnometry	3.95		BASF
Surface charge	IEP		IEP	-46		BASF	IEP			
Surface charge	zeta-potential pH7	mV	zeta-potential at pH7 in 10 mmol/l KCl water	3.2		BASF	zeta-potential at pH7 in xxx water	-17		BASF
AUC: size	D50 number metric (nm)	nm	D50 number metric	78		BASF	D50 number metric	15		BASF
Water contact angle: surface hydrophobicity	Water contact angle	°	sessile drop: MilliQ water	25°		BASF	sessile drop: MilliQ water	60°	1.8	nanoGRAVUR, BASF

d. RESULTS ON ENDOTOXIN TESTING (ALL MATERIALS)

Endotoxin (also known as lipopolysaccharide (LPS)) is a molecule found in the outer membrane of Gram-negative bacteria. It can initiate a strong immune response and serves as an early warning signal of bacterial infection (Pålsson-McDermott and O'Neill 2004). The Food and Drug Administration (FDA) recommends a limit of 0.5 EU/ml for medical devices (FC 2012). As endotoxin can easily bind to the surface of ENM it is important to include endotoxin tests since the presence of LPS in the ENM suspensions can result in an induction of inflammation, *i.e* false positive signals. AMI provided a SOP for undertaking the endotoxin test evaluation (Annex III). The Pierce LAL Chromogenic Endotoxin Assay was used to test endotoxin presence in suspensions prepared with all Tier 1 materials following the NanoReg protocol (Fig. 2). None of the tested materials exhibited endotoxin levels above the FDA recommended limit of 0.5 EU/mL. There is a false positive reading for Ag-NM300K and carbon nanotubes (MWCNT NM400 and NM402, Mitsui-7), which can be explained by the interference of material with the assay reagents. Particularly high interference was observed for Ag-NM300K due to the colour of the sample's solution, as endotoxin assays are colorimetric-based assays. Endotoxin evaluation of these ENM are currently repeated by including an additional test using the Endosafe Nexgen PTS device.

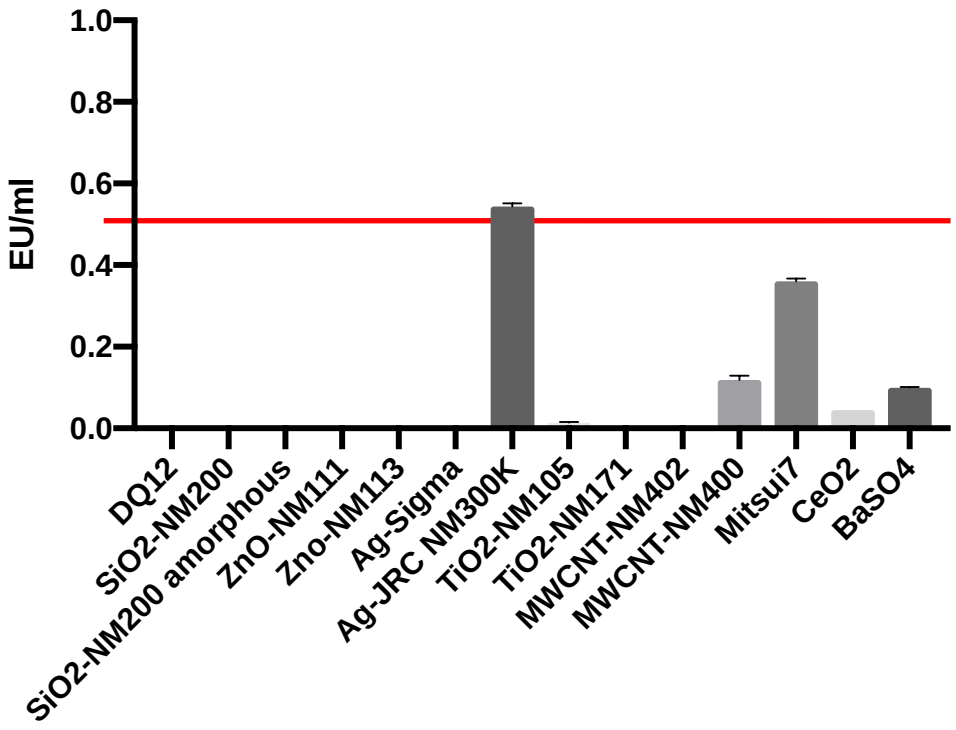

Fig. 2. Endotoxin concentration (EU/ml) for Tier 1 nanomaterials obtained using the Pierce LAL Chromogenic Endotoxin Assay

e. METHOD DOCUMENTATION OF MEASURED SAMPLES:

Crystalline phase by XRD

KRISS utilized a Rigaku SmartLab for the XRD analysis of the following samples: ZnO NM111, TiO_2 NM105, amorphous SiO_2 IUF, Ag Sigma 576832, Ag NM300, MWCNT NM402, ZnO NM113, MWCNT NM400, TiO_2 E171. Powder samples were loaded on 20mmX20mm glass sample holder. After inserting the sample, the z-position of sample stage is controlled so that incident X-rays can enter centre of the sample. The X-ray is generated by rotating anode X-ray generator of Cu. We executed a 2theta-theta coupled scan from 10deg to 100deg with step width of 0.02deg and a second duration time per step. Measured data is refined by Rietveld analysis using PDXL from Rigaku. XRD analysis were performed following an in-house KRISS protocol, which was established according to suggested requirements in ISO 17025 (General requirements for the competence of testing and calibration laboratories), and technically to JIS K 0131 (General rules for X-ray diffractometric analysis) and BS EN 13925-4 (Non-destructive testing X-ray diffraction from polycrystalline and amorphous materials).

Impurity detection by WDXRF

NRCWE conducted semi-quantitative elemental analysis on powder samples using a Bruker S8 Tiger wave length dispersive X-ray fluorescence (WDXRF) spectrometer using Rh X-ray source operated at 60 kV. Powdered samples of 2–5mg were placed on a XRF thin film (mylar sheet with a thickness of 6 µm), which was fixed in a 40 mm diameter sample cup (Fluxana, Kleve, Germany). The measurement time was 17 min. Results were manually post-processed for each element individually, to account for low concentrations and peak overlaps.

Coating detection by TGA/DTG

Thermogravimetric Analysis Mass Spectrometry (TGA-MS) data was provided from NRCWE on all Tier 1 and 2 materials apart from $BaSO_4$ JRCNM50001a and amorphous SiO_2 IUF. For analysis of materials of interest, NRCWE used a fast TGA screening method which runs from 25 °C with 10 °C /min up to a 1000 °C. The SOP for the screening method is described in ANNEX IV.

The more detailed and slower TGA analysis of selected materials used the NANoREG procedure described in the NANoREG D2.4 “protocol for quantitative analysis of inorganic and organic MNM surface coatings” . Mass losses below 100 °C was considered ascribed to adsorbed water and any mass loss above 100 ° was ascribed to potential presence of organic coatings or carbon in the case of CNT. It should be noted that mass-losses at higher temperatures may also be due to degradation of associated organic compounds, inorganic impurity compounds (e.g., sulphates, hydroxides), and de-hydroxylation etc. In this case, presence of organic coatings were anticipated when specific mass-losses were clearly observed within relevant temperature intervals. Further chemical analysis is required for identification of the chemical substances when a relevant high mass-loss has been identified.

The TGA-MS was carried out on Netzsch STA 449F3 coupled with gas transfer line to QMS D Aëolos mass spectrometer. Two TGA-MS methods were used: a screening method for fast determination of materials mass loss and a more detailed method. The fast screening method starts at 25 °C and ends at 1000 °C with a heating rate of 10 °C/min (duration 97.5 min). The ambient air flow was 40 ml, with balance flow of 60 ml of nitrogen. The more detailed method was performed as described in the NANoREG D 2.4 for “protocol for quantitative analysis of inorganic and organic MNM surface coatings” as follows: Step 1. Heating from room temperature up to 50 °C at rate of 10 °C/min. Step 2. Hold for 1 min. at 50 °C Step 3. Heating up to 100 °C at rate of 2.5 °C/ min. Step 4. Hold at 100 °C for 10 min. Step 5. Heating up to 800 at rate 2.5 °C/min. Step 6. Hold at 800 °C for 1 min. Step 7. Cooling down to room temperature. The duration of this detailed method is 318 min.

Size and shape analysis by TEM and SEM

ISTEC-CNR performed morphological analysis and particle size distribution on MWCNT NM402, Ag NM300, crystalline SiO_2 IOM, amorphous SiO_2 IUF, Ag Sigma 576832, ZnO NM111 samples using a FEI Tecnai F20 microscope operating at an acceleration voltage of 200 kV. TEM samples were prepared following NanoDefine “Protocols for sample carrier preparation and depositions of samples” (D2.8). About 50 µL of diluted STOCK suspension (256 ppm) were deposited on grid (300 mesh; holey carbon film on copper grid) and dried in air. Image analysis was performed for each sample to calculate the particle size distribution, average diameter and standard deviation.

Moreover, morphological analysis and the particle size distribution on TiO_2 E171 and ZnO NM113 samples were carried out by Field Emission Scanning Electron Microscopy (FESEM) analysis using Zeiss Sigma microscope at 4kV with a working distance of 3.7mm using the InLens detector. About 100 µL of diluted STOCK suspension (256 ppm) were deposited on silicon wafer, dried in air and then treated at 100°C for 5 minutes under irradiation of IR lamp. In turn, the wafer was fixed on a standard aluminium SEM stub using conductive adhesive tape. The sample, as prepared, were sputter-metallized with gold (thickness = 2 nm).
Image analysis was performed for each sample to calculate the particle size distribution, average diameter and standard deviation. Automated image evaluation was conducted by using ImageJ plugin (https://imagej.net/ParticleSizer) and the NanoDefine SOP for the particle sizer (not public) was used by BASF for the estimation of particle size and shape.
Additionally, BASF performed size and shape analysis on CeO_2 NM212 and $BaSO_4$ NM220 using a Tecnai G2-F20ST machine (FEI Company, Hillsboro, USA) operated at 200 keV. Images and spectroscopy data were evaluated following NanoDefine SOP and using the Olympus (Tokyo, Japan) iTEM 5.2 (Build 3554) and FEI TIA 4.1.202 software package.

Surface Composition by XPS
KRISS performed XPS analysis with a VersaProbe II Spectrometer (Ulvac-Phi, Japan) on the following samples: CeO_2 NM212, $BaSO_4$ NM220, ZnO NM111, TiO_2 NM105, amorphous SiO_2 IUF, Ag Sigma 576832, Ag NM300, MWCNT NM402, ZnO NM113, MWCNT NM400, TiO_2 E171. The instrument was calibrated by clean pure Au and Cu foils. Measured values for electron binding energies (BE) were 84.00 ± 0.02 eV, and 932.00 ± 0.05 eV. The samples were irradiated with monochromatic Al Kα X-rays (ħω=1486.6 eV, 25 W) using X-ray spot size of 100x100 μm^2 and a take-off angle (TOA) of 45 º with respect to the sample surface. The base pressure of the instrument was better than $1x10^{-9}$ Torr and the operating pressure better than $3x10^{-9}$ Torr. Electron and ion guns were used to compensate for surface charging and all spectra were corrected by setting hydrocarbon at BE=285.00 eV. Each survey spectrums (0-1000 eV for E 171 and 0-1200 eV for the other samples) were recorded at pass energy (PE) of 93.9 eV. In addition, each set of high-resolution spectra (PE=23.5 eV for E171 and PE=46.95 eV for the other samples) was also recorded with step size of 0.1 eV, from which surface chemical compositions (at %) were determined by relative atomic sensitivity factors (RASFs). Samples were not etched or pre-treated before each measurement. Most samples were supplied in powder form, but, the Ag NM300 was suspension in liquid so it was dried in air for measurement, while MWCNT NM400 and NM402 was compressed into pellet. Atomic composition is calculated from the average values of the three measurements. These measurements were performed following ISO 16243 (recording and reporting data in XPS) and ISO 10810 (XPS guideline for analysis).
On the other hand, crystalline SiO_2 DQ12 IOM was analysed by BASF. The XPS analysis was carried out with a Phi Versa Probe 5000 spectrometer using monochromatic Al Kα radiation (49 W). The instrument work function was calibrated to give a binding energy (BE) of 84.00 eV for the Au 4f7/2 line of metallic gold and the spectrometer dispersion was adjusted to give a BE of 932.62 eV for the Cu 2p3/2 line of metallic copper. Survey scan analyses were carried out with an analysis spot of 100x1400 μm^2 area, a pass energy of 117 eV and an energy step size of 0.5 eV. High resolution analyses were carried out on the same analysis area with a pass energy of 23.5 eV and an energy step size of 0.1 eV. Spectra have been charge corrected to the main line of the carbon 1s spectrum set to 284.5 eV as a typical value quoted for the energy of the peak of aromatic carbon.
C1s-Spectra were analyzed using Casa-XPS software (2.3.17ed., Casa Software Ltd.) using Shirley background subtraction in the energy region of 280-298.5 eV. Sp^2-hybridized carbon was described by a Lorentzian asymmetric line shape with tail damping as provided by the software with an asymmetry index of 0.0913 allowed to vary its FWHM in the range of 0.7 to 1.3eV. All other peaks were fitted using a voigt function of the form LF(1,1,900) as provided from the software accounting for the presence of functional groups (Hydroxyl, carboxyl, Epoxy, as well as resonances from the aromatic system (shake-up structures and plasmon resonances) as given in the literature (Leiro et al. 2003). Relative sensitivity factors as provided by the instrument manufacturer were used for quantification.

Surface area by BET
BASF measured the surface area of the following samples: CeO_2 NM212, $BaSO_4$ NM220, ZnO NM111, amorphous SiO_2 IUF, crystalline SiO_2 DQ12 IOM, SiO_2 NM200, Ag Sigma 576832, MWCNT NM402, ZnO NM113, TiO_2 E171.

Specific surface area was determined with the BET method using a Micromeritics Gemini V. Samples were degassed at 100°C under vacuum for 30 min. Nitrogen adsorption isotherms at 77 K were recorded at five pressures between 0.05 and 0.3 P/P0. Measurements were performed adhering to the standard DIN ISO 9277-2014-01.

Density by He pycnometer

Skeletal density of all Tier 1 and 2 materials (apart from $BaSO_4$ JRCNM50001a) was determined by BASF using a He pycnometer (Micromeritics AccuPyc II 1340). The sample amount was between 0.2-2.0 g without pretreatment. Samples were measured at 20 °C, applying ten He purging cycle of the chamber before the measurement. Samples were analyzed according to DIN EN ISO 1183-3. Ag NM300 was not measured because it is a water suspension.

Surface charge by Z Potential

Besides $BaSO_4$ JRCNM50001a, BASF measured the surface charge of all Tier 1 and 2 ENM. The zeta potential and IEP measurements were carried out at room temperature (25 °C) as a function of pH using a zeta potential analyzer (Malvern Zetasizer Nano ZS). Each zeta potential value was calculated in an average of 22-30 runs at pH 7 in 10 mM KCl water solution. Each IEP value was determined by carrying out measurements in the pH range from 3 to 10. The measurements were started at the pH that the samples displayed after dilution. The pH was adjusted by HCl or NaOH. The samples were measured in 10 mM KCl water solution.

Mean diameter by AUC

Size distribution of particles was provided by BASF using an AUC-Beckman XL centrifuge with ramped speed from 1,000 to 15,000 rpm equipped with an interference optical system. Samples analyzed were: TiO_2 NM105, amorphous SiO_2 IUF, crystalline SiO_2 IOM, Ag Sigma 576832, Ag NM300, MWCNT NM402, MWCNT NM400, SiO_2 NM200, TiO_2 E171. A refractive index detector was synchronized to the rotation of the centrifuge, to enable observation of the colloidal speed of migration during centrifugal separation. Specimens were analyzed according to project NanoDefine 2017 and previous published work (Mehn et al. 2018).

Surface hydrophobicity by Water contact angle

The material hydrophilicity of $BaSO_4$ NM220, CeO_2 NM212, amorphous SiO_2 IUF, Ag Sigma-576832, MWCNT NM402, ZnO NM111, TiO_2 NM105, Crystalline SiO_2 DQ12, Ag NM300, TiO_2 E171 was evaluated by a water contact angle measurement using Drop Shape Analyzer - DSA100. Sample powder (~ 0.5 g) was spread as a thin layer on the surface of the sticky sample holder by pressing the surface with a spatula. The sample holder is a 3M Color Laser Transparency Film plate covered with a homogenous adhesive layer (0.25 mm) of Acronal® V 215. Therefore, a nitrogen gun is used to gently blow the powder residuals not attached to the sample holder's surface. Finally, contact angle measurement was performed at 23 °C by measuring the diameter of the spherical crown of 2 µL water dropped on the surface of sample layer. The measurement was performed following the KRÜSS Technical Note TN306e, C. Rulison, 1999.

References of data that was not re-measured but included from existing reports:

XRF and AUC data for $BaSO_4$ NM220 was respectively extracted from previous works (Konduru et al. 2014) (Wohlleben et al. 2013).

The Konduru et al. article was also exploited for the complete characterization (XRD, XRF, TEM, XPS, BET, Z-pot) of $BaSO_4$ JRCNM50001a characterization.

XRF, TEM and BET analysis of TiO_2 NM105 was imported from literature (Arts et al. 2016).

Such aforementioned scientific paper (Wohlleben et al. 2013) was also used for the XRD characterization of Crystalline SiO_2 DQ12.

MWCNT NM400 data collection was completed importing TEM values from JRC report (Rasmussen et al. 2014) and BET and water contact angle data from NanoGRAVUR project.

JRC report (Rasmussen et al. 2013) and ECETOC project were exploited to provide respectively XRD, TEM, XPS data and water contact angle for SiO_2 NM200.

Regarding MWCNT Mitsui-7, XRD, TEM and BET were extracted from the previous project Nanogenotox and BET data were obtained from a published NRCWE scientific work (Jackson et al. 2015).

f. BIBLIOGRAPHY

Arts, Josje H. E., Muhammad-Adeel Irfan, Athena M. Keene, Reinhard Kreiling, Delina Lyon, Monika Maier, Karin Michel, Nicole Neubauer, Thomas Petry, Ursula G. Sauer, David Warheit, Karin Wiench, Wendel Wohlleben, and Robert Landsiedel. 2016. 'Case studies putting the decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping) into practice', *Regulatory Toxicology and Pharmacology*, 76: 234-61.

FC, Omalleys 2012. 'Guidance for Industry Pyrogen and Endotoxins Testing: Questions and Answers ', *FDA Guidance Document*: 1-13.

Jackson, Petra, Kirsten Kling, Keld Alstrup Jensen, Per Axel Clausen, Anne Mette Madsen, Håkan Wallin, and Ulla Vogel. 2015. 'Characterization of genotoxic response to 15 multiwalled carbon nanotubes with variable physicochemical properties including surface functionalizations in the FE1-Muta(TM) mouse lung epithelial cell line', *Environmental and Molecular Mutagenesis*, 56: 183-203.

Konduru, Nagarjun, Jana Keller, Lan Ma-Hock, Sibylle Gröters, Robert Landsiedel, Thomas C Donaghey, Joseph D Brain, Wendel Wohlleben, and Ramon M Molina. 2014. 'Biokinetics and effects of barium sulfate nanoparticles', *Particle and fibre toxicology*, 11: 55.

Leiro, J. A., M. H. Heinonen, T. Laiho, and I. G. Batirev. 2003. 'Core-level XPS spectra of fullerene, highly oriented pyrolitic graphite, and glassy carbon', *Journal of Electron Spectroscopy and Related Phenomena*, 128: 205-13.

Mehn, Dora, Iria Maria Rio-Echevarria, Douglas Gilliland, Michael Kaiser, Klaus Vilsmeier, Peter Schuck, and Wendel Wohlleben. 2018. 'Identification of nanomaterials: A validation report of two laboratories using analytical ultracentrifugation with fixed and ramped speed options', *NanoImpact*, 10: 87-96.

Ministère de l'Environnement, de l'Énergie et de la Mer. 2015. 'Éléments issus des déclarations des substances à l'état nanoparticulaire: Exercice 2015'.

Pålsson-McDermott, Eva M., and Luke A. J. O'Neill. 2004. 'Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4', *Immunology*, 113: 153-62.

Rasmussen, Kirsten , Jan Mast, Pieter-Jan De Temmerman, Eveline Verleysen, Nadia Waegeneers, Frederic Van Steen, Jean Christophe Pizzolon, Ludwig De Temmerman, Elke Van Doren, Keld Astrup Jensen, l Renie Birkeda, r Axel Clausen Pe, Yahia Kembouche, Nathalie Thieriet, Olivier Spalla, Camille Giuot, Davy Rousset, Olivier Witschger, Sebastian Bau, Bernard Bianchi, Boris Shivachev, Louiza Dimowa, Rosita Nikolova, Diana Nihtianova, Mihail Tarassov, Ognyan Petrov, Snejana Bakardjieva, Charles Motzkus, Guillaume Labarraque, Caroline Oster, Giulio Cotogno, and Claire Gaillard. 2014. 'Multi-walled Carbon Nanotubes, NM-400, NM-401, NM-402, NM-403: Characterisation and Physico-Chemical Properties', *EUR - Scientific and Technical Research Reports*, JRC91205.

Rasmussen, Kirsten, Agnieszka Mech, Jan Mast, Pieter-Jan De Temmerman, Nadia Waegeneers, Frederic Van Steen, Jean Christophe Pizzolon, Ludwig De Temmerman, Elke Van Doren, Keld Astrup Jensen, Renie Birkedal, Marcus Levin, Signe Hjortkjaer Nielsen, Kalevi Koponen Ismo, Per Axel Clausen, Yahia Kembouche, Nathalie Thieriet, Olivier Spalla, Camille Giuot, Davy Rousset, Olivier Witschger, Sebastian Bau, Bernard Bianchi, Boris Shivachev, Douglas Gilliland, Francesca Pianella, Giacomo Ceccone, Giulio Cotogno, Hubert Rauscher, Peter Gibson, and Hermann Stamm. 2013. 'Synthetic Amorphous Silicon Dioxide (NM-

200, NM-201, NM-202, NM-203, NM-204): Characterisation and Physico-Chemical Properties', *EUR - Scientific and Technical Research Reports*.

Wohlleben, W., L. Ma-Hock, V. Boyko, G. Cox, H. Egenolf, H. Freiberger, B. Hinrichsen, S. Hirth, and R. Landsiedel. 2013. 'Nanospecific guidance in REACH: A comparative physical-chemical characterization of 15 materials with methodical correlations', *J.Ceram.Sci.Tech.*, 4: 93-104.

3. Deviations from the Workplan

As an adaptation to availability of lab resources, XRD characterization was performed by KRISS institute instead of NRCWE.

4. Performance of the partners

All partners have fulfilled their tasks required to support this deliverable in satisfactory time and have provided high-quality, robust data-sets.

5. Conclusions

The Steering Board deems this deliverable to be fulfilled satisfactorily.

6. Annexes:

ANNEX I: Guidance Doc 1_Test Materials and Guidance on PATROLS NIWO-tool_Final

ANNEX II: Documentation of Tier 2 selection:

For ecotox models: the Dec 2018 General Assembly at Faenza decided Tier 2 ENM were not currently need due to the volume of work required to fully evaluate the Tier 1 ENM and that at this time, there was no value to add further testing materials. T1.1 will however review this position with WP5 again at November 2019 General Assembly.

For human lung/liver models: vivid discussion towards the selection of Tier 2 ENM were held during the Dec 2018 General Assembly at Faenza. The summary of this discussion was:

1. Agreement on criteria to select Tier 2 materials;
2. Collecting ad hoc proposals on sticky notes (25 received, transcripted in minutes);
3. Asking for email contributions (received none);
4. Iterated proposal once with Steering Board, revised, then in March, the following ENM were approved by the Steering Board:

Material	Systematic variation vs tier 1	Industrial / societal relevance, e.g. registered in R-nano	Existing benchmark data INHALATION	Existing benchmark data ORAL	Further notes
MWCNT, specifically NM400 (=NC7000)	compares rigid (Mitsui) vs flexible (NM400)	Low tonnage	**Inhalation** 90d	??	Challenge the rigidity hypothesis
TiO2 E171 +TiO2 pigment grade	Compares NM105 nano vs E171 non-nano	High tonnage	 **Inhalation** 90d, 180d	**Oral** (KRISS, new 90d) + many further studies	KRISS sources E171, ISTEC subsamples To be sourced
ZnO NM113	Compares non-nano vs. nano (NM110)	Significant tonnage	**Inhalation** STIS	**Oral** (acute OECD 423)	Establish assays to differentiate ion-vs-particle
SiO2 NM200 (and Levasil 200)	Differ vs. silica_IUF in dispersability, solubility	High tonnage	**Inhalation** 90d 2007	**Oral** 2-generation 2015 (**Oral** OECD 407)	Already included via guidance

This election was made during meetings and teleconferences in accordance with PATROLS transparent criteria. The rationale behind this proposal was to be able to compare (a) rigid vs flexible MWCNT, (b,c) nano vs non-nano particles and (d) different NM dispersibility.

ANNEX III: PATROLS SOP Endotoxin

ANNEX IV: TGA Screening SOP

Guidance Document 1 for Grant Agreement Number 760813

Test Materials and Guidance on the use of the PATROLS Material Information and Web-Order Tool
Version 1.0

Steering Board submission date: 27/04/2018

Lead beneficiary for this document: *NRCWE*

Dissemination Level:		
PU	Public	
PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including the Commission Services)	
CO	Confidential, only for members of the consortium (including the Commission Services)	CO

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No. 760813.

TABLE OF CONTENTS

1. Description of task

This document is created to instruct the PATROLS partners on:

1) Which test materials have been selected for use in the project
2) How to order the test materials using the PATROLS centralised web-order site.
3) How to retrieve physicochemical information on the test materials using the PATROLS centralised web-order site

The work is a result of initial work in Task 1.1. The work is specifically related to the section in Task 1.1 describing: "Tier 1 OECD ENM sub-sampled or newly synthesised material sub-samples will be accessible from JRC-Ispra and the Fraunhofer Institute or in some cases commercially available. Relevant NanoDefine materials will be sourced from the NanoDefine archive at JRC-Geel (also as sub-sampled and characterised ENM). All sample ordering will be managed through the nanomaterial web-order system established as part of the EU FP7 NANoREG, which directly links "customers" and different material providers for easy order management. …. … … Technical data sheets will be provided and updated monthly for direct access via the nanomaterial web-order system established by the NRCWE during the NANoREG project and entries will be exportable to the PATROLS database in WP6."

2. Description of work & main achievements

In accordance with the Task 1.1 description, the PATROLS Nanomaterial Information and Web-Order site was developed as a service to the PATROLS partners to create a simple centralised site for:

1) Ordering the test materials to be used in the PATROLS project.
2) Obtaining updated information on the physicochemical characteristics of the test materials.

The PATROLS web-order site was modified from the NANoREG tool to suit better the PATROLS project needs and new information exchange requirements requested by the material suppliers. The modifications involved in particular, redesign to represent PATROLS and on request that the material providers do not need to maintain the orders through the web-order facility. This means that the responsibility on follow-up lies completely with the "costumers" and test material providers. The number of orders and content can still be monitored by the NRCWE as the daily manager of the tool. The actual web-design and programming was, based on cost-benefit, done using the same consultant as developed and maintained the NANoREG web-order site.

The primary suite of Tier 1 test materials had already been pre-selected as part of the application stage and listed in the 760813 Annex 1 Description of Action Part (A). An important fraction of these materials originated from the past so-called Sponsorship Test programme organized by the OECD Working Party on Manufactured Nanomaterials and new analogue batches produced by the Nanomaterial repository at JRC Directorate F – Health, Consumers and Reference Materials, Ispra (Italy) and linking to large in vivo studies such as the German lead 90-day $BaSO_4$ and CeO_2 inhalation studies, which were

conducted as part of the EU FP7 NANoREG Project.

Unfortunately, discussions with the staff at the Nanomaterial repository at JRC revealed that they did not have all test materials in sufficient stock. In other cases JRC could not ensure that stock supply would be sufficient to cover the further defined needs of the PATROLS project. Therefore, major parts of the original OECD WPMN test materials are only available from the Fraunhofer Institute for Molecular Biology and Applied Ecology (IME), Schmallenberg (Germany). Fraunhofer IME can deliver all original OECD WPMN materials and also confirmed to have additional stock of all these test materials if needed. The only difference between receiving material from the JRC and the Fraunhofer IME is that all materials provided by Fraunhofer IME will be at cost 25€ per sample vial.

Regarding new materials, it was attempted to come to an agreement on subsampling of these additional test materials by the JRC (IUF Silica: Sigma Aldrich silica; Sigma, S5130, Mitsui-7: NRCWE-007, Mitsui Ltd and a newer Mitsui-7 batch: Hodogaya Chem). Unfortunately, it has not been possible to verify that these new materials can be sub-sampled at the JRC at this point in time. The contingency plan is to inquire Fraunhofer IME about the potential costs to subsample the additional test materials in their facility. An update of Guidance 1 will follow when these final subsampling and distribution issues have been solved.

3. PATROLS test materials and ordering

3.1 Selected test materials

PATROLS has been designed to test a group of pre-defined (first-wave) materials and a later supplemental suite of materials to be defined in WP1 for use around Month 18 in the project. Table 1 lists the currently selected first wave test materials.
Except for DQ12, all selected test materials can be ordered at the PATROLS web-order and material information site (http://www.patrols-materials.eu/). An order can be placed centrally by the user from the web-order site to each of the individual sample providers (JRC and Fraunhofer) in one single operation; independent of who is providing the test material. Further details are given in section 2.2. DQ12 will be distributed directly to by IOM in Edinburgh.

3.2 Guidance for use of the test materials information and web-order site

All available PATROLS test materials can be ordered through the PATROLS Test materials Information and Web-Order system. It can be reached directly using this link: http://www.patrols-materials.eu/. The site gives general information on the material data and in time, will also function to offer up-to-date information in Technical Data Sheets (TDS) on the key physico-chemical data on each of the MNMs.
At least one person from each institute that need to order test materials and/or retrieve test material information, will have to register. When registered, the user cannot access the site before they have been accepted by the site manager at NRCWE. Please click on the appropriate field for user registration on the landing site or follow the link directly here to register: http://www.patrols-materials.eu/Account/Register. The review of the user profile is established due to practical experience, to reduce the risk of disruption.

Table 1. The PATROLS test materials.

Type of MNM	Material Identification codes	Cost [€]	Provider
Synthetic Amorphous Silica	NM-200 (sonicator calibration material) IUF-silica (Sigma Aldrich, Sigma, S5130)	25 0	Fraunhofer Await[£]
Quartz	DQ12[*]	0	IOM
Titanium Dioxide	NM-105 JRCNM01005a (alias NM-105)	25 0	Fraunhofer JRC
Zinc Oxide	NM-110[€] JRCNM01100a (alias NM-110)[€] JRCNM62101a (new NM-110 batch)[€] NM-111 JRCNM01101a (alias NM-111)	25 0 0 25 0	Fraunhofer JRC JRC Fraunhofer JRC
Cerium Dioxide	NM-212 JRCNM02102a (alias NM-212)	25 0	Fraunhofer JRC
Barium Sulphate	NM-220 JRCNM50001a (new NM-220 batch)	25 0	Fraunhofer JRC
Silver	NM-300K NM-300K DIS NM-302 NM-302 DIS	25 25 25 25	Fraunhofer Fraunhofer Fraunhofer Fraunhofer
Multi-walled carbon nanotubes	NM-402 JRCNM04002a (alias NM-402) NRCWE-006 (Mitsui Ltd batch)[£] Mitsui-7 (new Hodogaya Chem batch)[£]	0 0 0 0	Fraunhofer JRC Await[£] Await[¥]

[*] Will be distributed directly from IOM to selected partners for use as specific reference material in certain tests. It will not be available in the we-order system. [€] In PATROLS this specific material will be used only for ecotoxicological studies and studies supporting these studies [£] Currently on hold. It is anticipated that these materials will be sub-sampled as JRC and enter the JRC repository. [¥] Will only be used by WP1 if received from Japanese colleagues. Splitting and distribution of this material awaits decisions.

Note 2.1: Due to negotiation with JRC (not partner in PATROLS), who runs a high-quality subsampling facility[1], the subsampling and final provider of IUF silica, NRCWE-006 (Mitsui Ltd. batch), and Mitsui-7 (Hodogaya Chem batch) is not yet resolved. The outstanding issues will be resolved as soon as possible.

When you have been accepted as user of the PATROLS test material information and order site, you can login and order your materials and retrieve state-of-the art technical data sheets on the test materials following the routes indicated in Figure 3. These Technical data sheets will be updated during the course of the PATROLS project as new and better data may be generated as part of the project.

1 http://publications.jrc.ec.europa.eu/repository/bitstream/JRC104369/jrc%20nanomaterials%20repository%20-%20technical%20report.pdf.

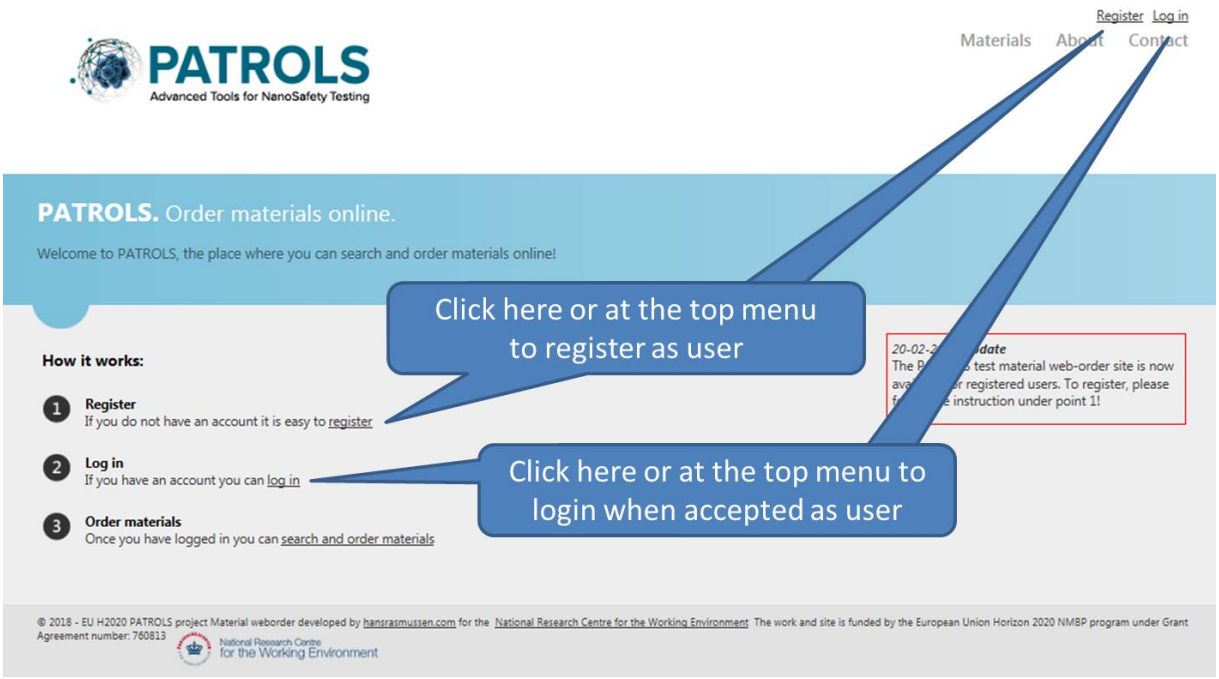

Figure 1. Please use the indicated entries to register and login at the PATROLS test material information and order site. See Figure 2 for guidance on filling out the registration page.

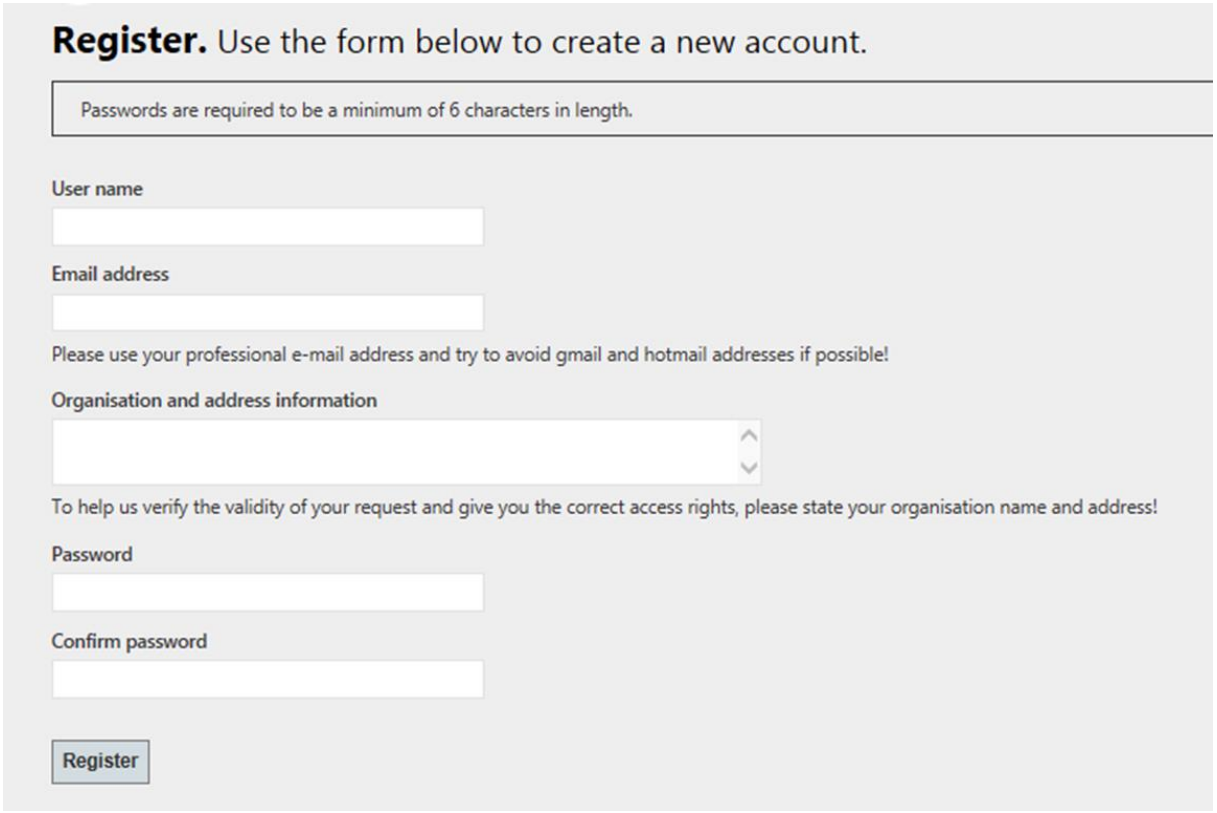

Figure 2. Fill out the registration and user information and submit your registration. Please remember to take note of your user name and password to login. PLEASE REMEMBER TO USE YOUR OFFICIAL WORKING E-MAIL, which will make it easier to identify you as an eligible user of the tool. When your user-profile is accepted, you will receive a confirmation on the e-mail used. Acceptance will typically happen within 24 hours on work days.

After ordering the materials, you will see the shopping cart page (Figure 4) from which you can adjust the number of vials needed and remove order lines as required. When finished, you press "Ready to order" after which you are requested to enter your billing and shipping addresses, including your VAT number.

Note 2.2: First time entering information, you may need to add your billing and shipping information even-though the address is checked out to be the same.

Upon submitting your order, each provider receives and e-mail on the order of materials that they provide and your shipping and billing information. You receive a conformation of your order by e-mail.

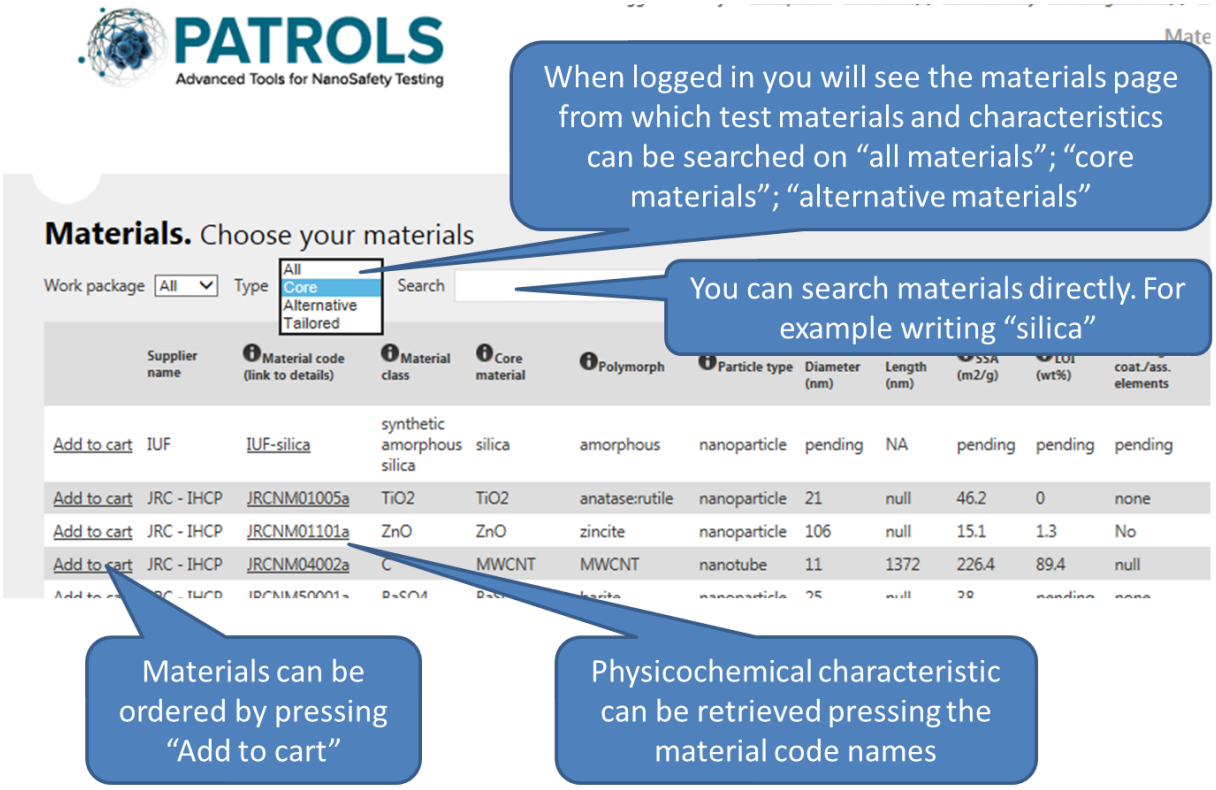

Figure 3. When logged in, you can retrieve information on the test materials, search test materials, and order materials for your laboratory following the routes indicated in the figure.

Figure 4. When pressing "Add to cart", the shopping card pops up and you can adjust the number of vials needed, delete order lines, and finally proceed to submit your order.

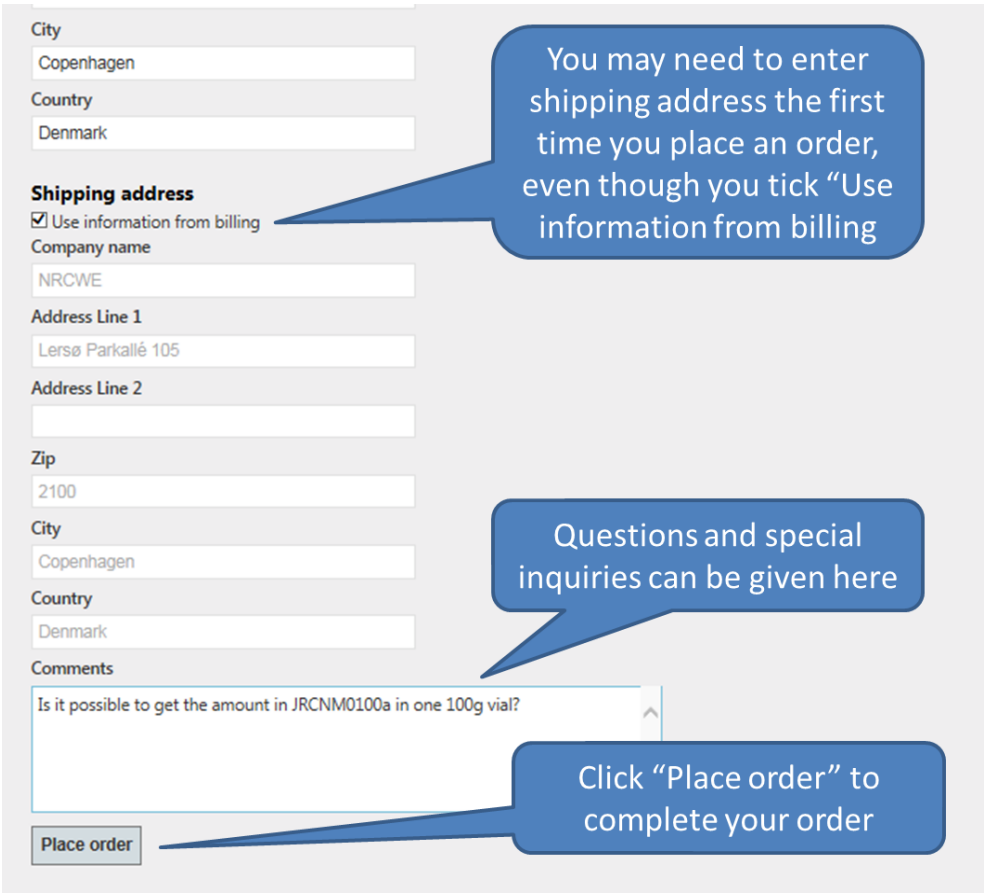

Figure 5. On the final order page, you enter the requested billing and shipment information. Mandatory information includes type of organization and your organizations VAT number. The VAT number is required for shipments from the Fraunhofer Institute where samples are purchased on a cost basis. Comments and questions can be passed on to the distributor in the "Comments" field. When all information is entered, you press "Place order" and the order goes out to the individual sample providers. Order confirmation will be send to your e-mail.

4. Deviations from the Workplan

There was no deadline specified for establishment of the PATROLS Nanomaterials Information and Web-Order site in the DOA. It was of high priority to establish the system as soon as possible and the expectation was to have it fully operational by middle of February. However, unforeseen delays were experienced due to different human resource and technical issues. In particular, the lack of formal agreement and resources at the JRC showed to be an obstacle for quick implementation. It is anticipated that most of the issues for Tier 1 materials have been solved by combining the Fraunhofer IME and JRC material stocks, but the solution for subsampling of new materials remains an issue to be solved in the near-future. An update of this document will follow when this issue is cleared. Periods of slow progress at NRCWE and at the IT-consultant in revising the web-tool was also experienced due to high work load.

5. Performance of the partners

NRCWE has been the only active partner in developing the PATROLS material information and web-order site and establishing the operational agreements for material supply from JRC, Ispra and Fraunhofer IME. NRCWE, ISTEC-CNR, SU and BASF have also handled information exchange on additional test materials from IUF (amorphous silica) and a new Hodogaya Chem batch (Mitsui-MWCNTs). SU, ISTEC-CNR and BASF have provided comments to the guidance document and dialogue for decision making.

6. Conclusions

The Steering Board deems this document to be satisfactory.

PATROLS
Advanced Tools for NanoSafety Testing

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 760813

PATROLS Standard Operating Procedures (SOP)

Guidance Document for Endotoxin Testing

This is a SOP used by members of PATROLS only

Adapted from the NanoImpactNet SOP, Clift *et al* (Deliverable 5.4 under the European Commission's 7th Framework Programme, Grant Agreement 218539).

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Version	Approval Date	Description of the change	Author(s) of change
1.0	24/09/2018	Initial Document	R. Lehner
1.1	23/10/2018	Adaption of the Document	R. Lehner
1.2	21/11/2018	Adaption to SOP	R. Lehner

Table of Contents

1 Introduction:

Endotoxin (also known as lipopolysaccharide (LPS)) is a molecule found in the outer membrane of Gram-negative bacteria. It can initiate a strong immune response and serves as an early warning signal of bacterial infection (Palsson-McDermott and O'Neill, 2004). The binding of LPS initiates the aggregation of different intracellular signaling proteins leading to cytokine production and the initiation of inflammatory signaling. Within this protein binding cascade, Toll-like-receptor 4 (TLR4) is the key receptor involved in LPS recognition and signal initiation in addition to the co-receptors CD14 and MD2 (Park and Lee, 2013). It has been shown that already very low levels of 0.1 EU/mL endotoxin can upregulate the expression of inflammatory genes (e.g. upregulation of the inflammatory interleukin-1β gene) in primary human monocytes (Lin et al., 2004, Oostingh et al., 2011). The Food and Drug Administration (FDA) redommends a limit of 0.5 EU/ mL for medical devices ((FDA), 2012). As endotoxin can easily bind to the surface of nanomaterials it is important to include endotoxin tests as described elsewhere (Li et al., 2015, Li and Boraschi, 2016) since the presence of LPS in the nanomaterial suspensions can result in an induction of inflammation that can be incorrectly attributed to the nanomaterial (Smulders et al., 2012, Li et al., 2017).

1.1 Scope and limits of the protocol

This guideline describes the endotoxin testing of nanomaterial suspensions intended for *in vitro* biological test systems by PATROLS. Depending on the physico-chemical characteristics of nanomaterials, such as the optical density, the interference of nanomaterial suspension with both the components and the detection readouts, which has to be considered for the planning of the experiments and the analysis.

2 Terms and Definitions:

Endotoxin

Part of the outer membrane of the cell envelope of Gram-negative bacteria

Note 1 to entry: The main active ingredient is lipopolysaccharides (LPS).

[SOURCE : ISO 29701:2010, definition 2.3]

Endotoxin unit (EU)
Standard unit of endotoxin activity

Note 2 to entry: The endotoxin unit was defined by the World Health Organization (WHO) Expert Committee on Biological Standardization (ECBS) in 1996, relative to the activity of 0,1 ng of WHO reference standard endotoxin (RSE) from *Escherichia coli* 0113:HK10:K(-) or 10 EU/ng

Note 3 to entry: EU is equal to international unit (IU) of endotoxin.
[SOURCE : ISO 29701:2010, definition 2.4]

3 Abbreviations:

LPS = Lipopolysaccharide
TLR4 = Toll-like-receptor 4
FDA = Food and Drug Administration
EMA = European Medicines Agency
LAL = Limulus Amoebocyte Lysate
RPT = rabbit pyrogen test
pNA = p-nitroaniline
EU/mL = endotoxin unit/mL

4 Principles of the Method:

Different assays to detect endotoxins have been described such as the rabbit pyrogen test (RPT) and the Limulus amoebocyte lysate (LAL) assay. These are the most commonly used endotoxin detection methods that are approved by the FDA and EMA, and also accepted by almost any other country. Due to the high cost and long execution time of the assay in combination with the need of using animals, RPT is now mainly applied in combination with the LAL test for analyzing parenteral drugs during the earlier development phase in biomedical research. For most other research fields, the most often used endotoxin detection method applied is the LAL assay since it represents a fast, sensitive and reasonably specific test method.

Therefore, endotoxin contamination of the materials used by the PATROLS WP partners will be assessed following the Limulus Amoebocyte Lysate (LAL) assay. The LAL assay is originally based on the blood cell extract of the horseshoe crab (a marine arachnid), for LPS endotoxin testing. There are three major types of basic LAL tests: gel-clot, turbidimetric and chromogenic. Dobrovolskaia et al. have declared that none of the currently available LAL formats is optimal for endotoxin assessment for nanomaterial testing and suggested that at least two LAL formats with different endpoints/readouts should be used (Dobrovolskaia et al., 2014).

The gel-clot assay is the simplest LAL test and is used to detect the presence or absence of endotoxin by either forming a detectable gel-clot (presence of endotoxin) or not. The assay is based on the initiation of a series of enzymatic reactions after encountering with the endotoxin. The activation of this pathway results in the production of at least three serine protease zymogens: Factor C, Factor B, and a proclotting enzyme. These enzymes alter the amoebocyte coagulogen present in LAL to form a detectable gel-clot. However, the test can be used in a qualitative and only semi-quantitative manner. In addition it has been shown that the gel clot LAL assay is not accurate for testing endotoxin contamination for different clinical-grade particles such as silica, silver, titanium dioxide, calcium carbonate and others (Brooks et al., 2002, Smulders et al., 2012, Dobrovolskaia et al., 2014, Kucki et al., 2014).

The turbidimetric assay is a technique that uses the change in gel turbidity to detect the activation of LAL reagent induced by endotoxin. The cleavage products coalesce as a result of ionic interactions that occur after the cleavage and cause the reaction mixture to become turbid. The turbidimetric method is sensitive to suspended or turbid materials and does often result in false positive results.

The chromogenic test is an optical analysis method that allows for quantitative measurement of endotoxin through color changes. In the presence of endotoxin, the components of LAL are activated in a proteolytic cascade that results in the cleavage of a colorless artificial peptide substrate present in Pyrochrome LAL. Proteolytic cleavage of the substrate liberates p-nitroaniline (pNA), which is yellow and has an absorbance of 405 nm. The degree and rate at which light is absorbed is directly

proportional to the amount of endotoxin within the sample allowing quantitative data analysis. In addition, the chromogenic LAL assay showed higher sensitivity compared to the gel clot assay but might in addition show interference with the LAL readouts due to physico-chemical characteristics such as the optical density of the materials.

Out of the three different assays, the chromogenic testing system shows the easiest and fastest handling, higher sensitivity as well as qualitative and quantitative data outcome. For those reasons, the chromogenic endotoxin testing assays are recommended for the testing of the PATROLS nanomaterials. However, interference of the test material with the readouts of the chromogenic LAL assays needs to be taken into consideration."[1]

[1] As the LAL is an extract of blood cells from the Atlantic horseshoe crab, there have been issues reported with ordering LAL based kits due to this.

5 Description of the Method:

5.1 Pierce LAL Chromogenic Endotoxin Testing

The endotoxin concentration in a sample is measured using the Pierce LAL Chromogenic Endotoxin Quantitation Kit *via* a chromogenic signal generated in the presence of endotoxins. Samples are measured on a microplate absorbance reader at 405nm. A standard curve is created using the *E. coli* endotoxin signal at different concentrations to calculate endotoxin levels as low as 0.1 EU/mL, where one endotoxin unit/mL (EU/mL) equals approximately to 0.1 ng endotoxin/mL of solution. Protein and antibody samples can be assayed in about 30 minutes.

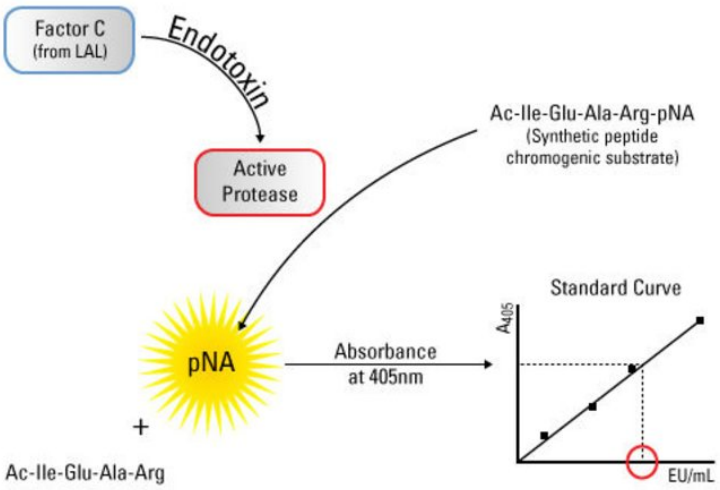

Figure 1: LAL Chromogenic Endotoxin Quantitation Kit reaction scheme. A small volume of the sample (10 µL) is combined with the LAL, and endotoxins in the sample activate the proteolytic activity of Factor C. When the chromogenic substrate is added, the activated protease catalyzes the cleavage of p-nitroalinine (pNA), resulting in yellow color that can be quantitated by measuring the absorbance at 405 nm (A405) and extrapolating against a standard curve.

5.2 Endosafe® negxen-PTS™ Chromogenic Endotoxin Testing

The Endosafe® negxen-PTS™ is a rapid, point-of-use handheld spectrophotometer that utilizes disposable cartridges for accurate real-time endotoxin testing. The user simply pipettes 25 µL of a sample into each of the four sample reservoirs of the cartridge. The reader draws and mixes the sample with the LAL reagent in the

sample channels in addition to the LAL reagent plus positive control. The sample is combined with the chromogenic substrate and incubated. After mixing, the optical density of the wells is measured and analyzed against an internally archived standard curve. By design, the cartridge technology automatically performs a duplicate sample/duplicate positive control LAL test.

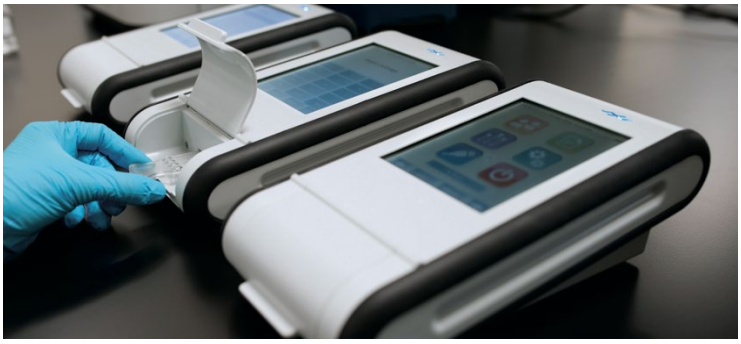

Figure 2: Portable Endotoxin Testing System

6 Materials and Instruments:

6.1 Pierce LAL Chromogenic Endotoxin Quantitation Kit

- Disposable endotoxin-free glass tubes or 1.5mL microcentrifuge tubes, pipette tips, 96-well microplates
- Heating block at 37°C ± 1°C
- Pipettor
- Microplate reader
- 25% acetic acid (Stop Reagent)

6.2 Endosafe® negxen-PTS™ device

- PTSTM Cartridges
- Endotoxin free reagent water
- Endoxotin free Dilution Tube
- Pipettor

7 Procedure:

Pierce LAL Chromogenic Endotoxin Testing:

1. Pre-equilibrate the microplate in a heating block for 10 min at 37°C ± 1°C
2. Incubate particles in endotoxin free water for 1 h at 37° C.
3. Spin down and collect supernatant (sample).
4. Dispense 50 µL of each standard or unknown sample replicate into the appropriate microplate well. Suitable controls need to be used to insure non-interference of the test material.
5. At time T=0, add 50 µL of LAL reagent to each well and incubate for 10 min.
6. After exactly T=10 min, add 100 µL of Chromogenic Substrate solution (prewarmed to 37°C±1°C) in the same order as the samples were added to each well. Incubate the plate at 37°C ± 1°C for 6 min.
7. At T=16 minutes, add 100 µL of Stop Reagent (25% acetic acid).
8. Measure the absorbance at 405 – 410 nm on a plate reader as soon as possible.
9. Subtract the average absorbance of the blank replicates from the average absorbance of all individual standards and unknown sample replicates to calculate mean ∆ absorbance.
10. Use the formulated standard curve (linear regression) to determine the endotoxin concentration of each unknown sample.

Endosafe® negxen-PTS™ Kinetic Chromogenic Endotoxin Testing:

1. Pipette 25 µL of a sample into each of the four sample reservoirs of the cartridge.
2. Perform the analysis.

7.1 Testing for nanomaterial interference:

Depending on the physico-chemical characteristics such as the optical density, the materials could interfere with the LAL readouts. In order to eliminate the interference, dilution of the sample is recommended (USP 30, 2012, (FDA), 2012, US Department

of Health and Human Services, 1987). If the endotoxin concentration of the tested sample is >1.0 EU/mL, dilute the sample five-fold in endotoxin-free water and subsequently re-test.

Assay inhibition occurs when substances in the test sample interfere with the LAL reaction. In the chromogenic assay, this inhibition results in a lower final absorbance, indicating lower levels of endotoxin than what may be present in the test sample. Determine the lack of product inhibition for each sample undiluted or at an appropriate dilution. To verify the lack of product inhibition, spike an aliquot or dilution of a test sample with a known amount of endotoxin (e.g., 0.5 EU/mL). Assay the spiked sample and the unspiked samples to determine the respective endotoxin concentrations. The difference between the two calculated endotoxin values should be equal to the known concentration of the spike ±25%.

References

(FDA), U. S. F. A. D. A. 2012. Guidance for Industry: Pyrogen and Endotoxins Testing: Questions and Answers. U.S. Food and Drug Administration (FDA).

BROOKS, R. A., J.A., W. & RUSHTON, N. 2002. Endotoxin contamination of particles produces misleading inflammatory cytokine responses from macrophages in vitro. *J Bone Joint Surg Br,* 84**,** 295-9.

DOBROVOLSKAIA, M. A., B.W., N., J.D., C., J.H., G. & MCNEIL, S. E. 2014. Choice of method for endotoxin detection depends on nanoformulation. *Nanomedicine (Lond),* 9**,** 1847-56.

KUCKI, M., C., C. & KRAEGELOH, A. 2014. Interference of silica nanoparticles with the traditional Limulus amebocyte lysate gel clot assay. *Innate Immun,* 20**,** 327-36.

LI, Y. & BORASCHI, D. 2016. Endotoxin contamination: a key element in the interpretation of nanosafety studies. *Nanomedicine (Lond),* 11**,** 269-87.

LI, Y., FUJITA, M. & BORASCHI, D. 2017. Endotoxin Contamination in Nanomaterials Leads to the Misinterpretation of Immunosafety Results. *Front Immunol,* 8.

LI, Y., P., I., E., C., F., T. N. & BORASCHI, D. 2015. Optimising the use of commercial LAL assays for the analysis of endotoxin contamination in metal colloids and metal oxide nanoparticles. *Nanotoxicology,* 9**,** 462-73.

LIN, S. M., C.W., F., O., K., M.M., W., K., B., S., M., V.A., W., A., S. & MARTIN, T. R. 2004. Differential regulation of membrane CD14 expression and endotoxin-tolerance in alveolar macrophages. *AM J Respir Cell Mol Biol,* 31**,** 162-70.

OOSTINGH, G. J., E., C., P., I., R., C., R., S., J., P., T., P., Y., K., D., O., F., F., H., L., D., L., F., R., I., N., H.., T., V.F., P., A., D. & BORASCHI, D. 2011. Problems and challenges in the development and validation of human cell-based assays to determine nanoparticle-induced immunomodulatory effects. *Part Fibre Toxicol,* 8.

PALSSON-MCDERMOTT, E. M. & O'NEILL, L. A. 2004. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology,* 113.

PARK, B. S. & LEE, J. O. 2013. Recognition of lipopolysaccharide pattern by TLR4 complexes. *Exp Mol Med,* 45.
SMULDERS, S., KAISER, J.-P., ZUIN, S., VAN LANDUYT, K. L., GOLANSKI, L., VANOIRBEEK, J., WICK, P. & HOET, P. H. M. 2012. Contamination of nanoparticles by endotoxin: evaluation of different test methods. *Particle and Fibre Toxicology,* 9**,** 41.
US DEPARTMENT OF HEALTH AND HUMAN SERVICES, U. F. 1987. Guideline on validation of the limulus amebocyte lysate test as an end- product endotoxin test for human and animal parenteral drugs, biological products, and medical devices.
USP 30. 2012. A harmonized standard for Bacterial Endotoxins Test. *USP 30,* NF 25.

NFA TGA Screening SOP

TGA-screening 97.5 min.

Preparation of sample

Analysis on the TGA is carried out in crucibles of alumina (Al_2O_2) with volume of 3.4 ml. The mass of the crucible and lit is weighed before pouring the chosen sample (either liquids or powders) into the crucible. This is to determine the mass of the chosen sample (see the table below: Safety and precaution).

Safety and precaution

Chemicals	Instuction for use	H/P sætninger	
Name of chemical and CAS-no.	Instruction from selling company or NFA instruction	List of Hazard and Precaution sentences	• Text of Hazard and Precaution sentences
TiO2 E171 NM-105 CAS-no.13463-677 Ag Sigma CAS-no.7440-20-2 NM-300K CAS-no.7440-20-2 NM-300K DIS NM-200 CAS-no.61790-53-2	PATROLS JRC Sigma Aldrich JRC JRC JRC	General precaution for all the materials Try not to create dust clouds Don't ingest Wear gloves Use eye	

This project is funded by the EU Framework 7 Programme, contract no 310584.

SAS(IUF) DQ12 NM-110 CAS-no.1314-13-2 NM-111 CAS-no.1314-13-2 NM-113 CAS-no.1314-13-2 NM-212 CAS-no.1306-38-3 NM-220 CAS-no.7727-43-7 NM-400 CAS-no.308068-56-6 NM-402 CAS-no.308068-56-6 Mitsui-7 CAS-no.308068-56-6	JRC JRC JRC JRC JRC JRC JRC JRC NRCWE	protection Don't spill the liquid For the most hazard of the materials use glove box.	

* MSDS (Material Safety Data Sheet) or NFA instruction (APBA)

Safety instructions

This project is funded by the EU Framework 7 Programme, contract no 310584.

Use proper fume hood with the necessary ventilation and filtering. Be careful not to create dust or drop liquids from the samples and follow the material safety data sheet instructions.

Starting the TGA-screening

1. Weighing the sample for the TGA-screening

Weigh the appropriate amount of material between 2-60 mg in the crucible within a fume hood with the proper ventilation and filters.

2. Transfer the crucible to the TGA

Transfer of the crucible is done with the lit on which is held down with a gloved hand so no overspill can occur during transfer. Then mount the crucible on the TGA weight and drive down the furnace.

3. Preparation of the TGA for screening

Choose the method called **TGA-screening.2** where the gas flow rate are purge gas 1 ambient air O_2/N_2 at 40 ml/min. and the balance protective gas 2 N_2 is set at 60 ml/min. The heat rate is 10^{o}C/min. heating to 1000^{o}C. Then make sure that the starting temperature is at correct temperature 25^{o}C and tarring the TGA weight to 0.000 mg and then press start. Now the TGA is running.

4. Ending of the TGA-screening for sample waste and disposal

- Wait until the crucible has cooled to room temperature.
- Transfer the crucible to a fume hood and use the proper waste disposal procedure for the given material that was analyzed.

5. Cleaning the crucible

The cleaning of the crucible is first done in a sonicator for 60 min. in deionized water and then following an organic solvent. The water and the organic solvent are properly disposed of in the waste disposal bin for solvent and water with chemical or material contaminates. The crucible is further cleaned in a furnace at 500 ^{o} C for evaporation of left over solvent or water species.

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