A common European approach to the regulatory testing of nanomaterials

Identification and optimization of the most suitable in vitro methodology
Deliverable 5.6

Introduction

The use of *in vitro* methods as part of a risk assessment strategy for nanomaterials has considerable potential with regards to reducing costs and lead-time. *In vitro* methods can (1) reduce the numbers of animals used in both research and risk/hazard assessments, (2) Mimic more reliably aerosol exposure, (3) investigate susceptible effects in disease models and (4) highly reduce experimental cost. However to be used in a regulatory context *in vitro* methods have to be validated in order to ensure they are predictive and reliable.

Task 5.5 of the NANoREG project evaluated the suitability of *in vitro* assays in connection to the *in vivo* experiments performed in this project. The results of this task are reported in Deliverable 5.06.

Description of Work

To detect potential contamination of the nanomaterials with endotoxin (a toxic substance that strongly can influence the results of *in vitro* assays), all nanomaterials ~~applied~~ used in this task have been tested for this substance. Characterisation and dispersion of NM was carried out following the NANoREG Guidance Document.

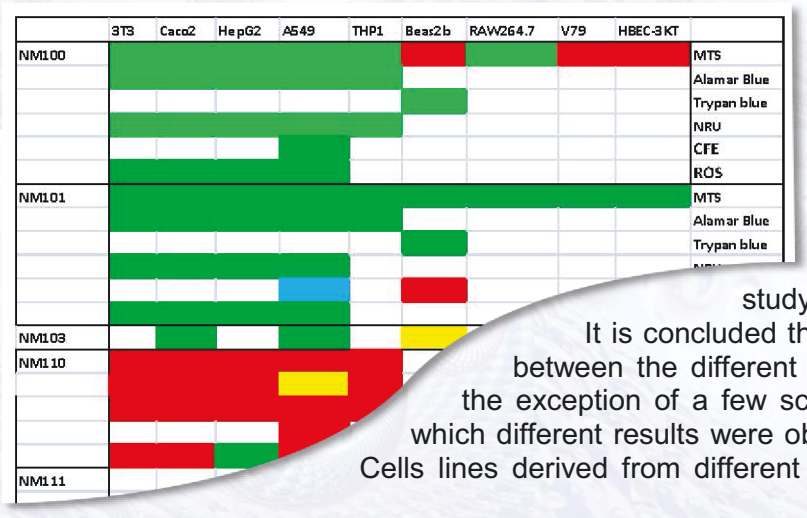
For cytotoxicity 6 different assays have been performed addressing cellular viability (MTS, Alamar blue, Neutral Red Uptake (NRU), Lactate Dehydrogenase (LDH), Colony forming efficacy (CFE) and Reactive Oxygen Species (ROS). For MTS a round-robin exercise has been performed.

Potential genotoxic effects have been addressed by 4 assays (Comet assay, Mouse lymphoma assay (OECD490), micronucleus assay (OECD 487) and cell transformation assay).

Immunotoxicity has been tested by a study on macrophage and monocyte cell lines (RAW 264.7 mouse cell line and THP-1 human cell line), evaluating NO production and pro-inflammatory cytokine secretion.

For several *in vitro* assays SOPs have been developed

Main results and evaluation



A comparison study for the 8 different basic toxicity methodologies. Red: IC50 <50 µg/mL, yellow: IC50 >50-100 µg/mL and green: no toxicity observed. Blue represents inconsistencies between partner results. White: not tested.

Among many other topics, the deliverable presents the results of a comparison study for different toxicity methods (see figure. It is concluded that -in general- there was good correlation between the different techniques for all NMs under study (with the exception of a few scattered cases and the NM400 series, for which different results were obtained depending on the technique used. Cells lines derived from different organs show different sensitivity towards

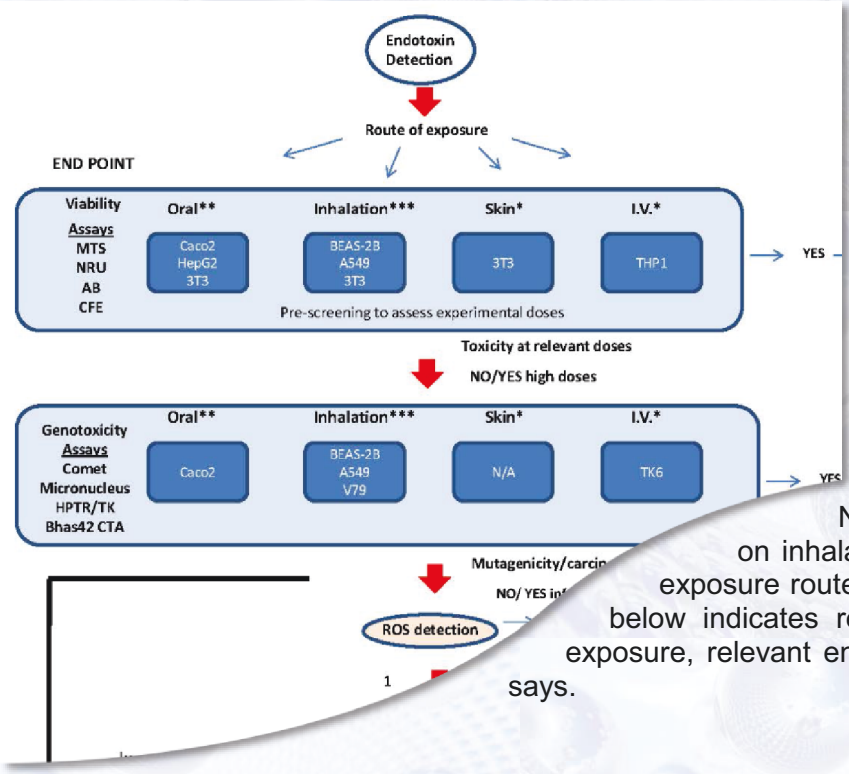
NM exposure. It is therefore important, while designing experiments, to select the cell line which best represents the intended exposure route.

The effect of different dispersion methods on the size distribution and toxicity of a number of NMs also has been detailed. A limited effect of the dispersion procedure was observed for some of the tested NPs: Sonicated SiO2 NM200 NPs and ZnO NM110 NPs induced a slightly higher toxic effect than stirred NPs. However, no significant impact of sonication was observed on the toxicity of Ag NM300K and NM302 NPs

	UAB	PToNANO	PToNANO	GAIKER	GAIKER	NILU	KI	ISS-INAIL	LEITAT	IEM	FIOH	CBT	STAMI	
NM100			A549				Beas-2b	Beas-2b						comet/comet-Fgp
			A549				Beas-2b	Beas-2b						micronudeus
NM101	Beas-2b		A549				Beas-2b	Beas-2b	Beas-2b			TK6		comet/comet-Fgp
	Beas-2b		A549				Beas-2b	Beas-2b						micronudeus
						V79								HPRT
NM103							Beas-2b							comet/comet-Fgp
							Beas-2b							micronudeus
NM110	Beas-2b			Beas-2b	Caco2					A549				comet
	Beas-2b													micronudeus
NM200	Beas-2b			Beas-2b	Caco2			Beas-2b		A549				
	Beas-2b							Beas-2b						
NM203	Beas-2b													
	Beas-2b													
NM212	Beas-2b		A549											
	Beas-2b		A549											
			A549											

The results of the genotoxicity tests reveal that most of the NMs are not genotoxic under the conditions of the study (consensus among different assays), except NM401 and NM402 that show genotoxicity in some of the cell types. In spite that comet and micronucleus assays are able to detect different types of genetic lesions, a good correlation between results obtained using these two methods was found. The exception was TiO2 (NM100 and NM101) for which comet produced a positive effect not observed by the micronucleus technique in 2 out of 3 partners. Overall the comet assay may represent a complementary test to the micronucleus assay. The comet assay allows for high-throughput adaptations (D5.07) and could potentially be included in the battery of in vitro tests for genotoxicity assessment of NMs.

The deliverable also presents results for inflammatory effects and immunotoxicity.



Based on the results of the *in vitro* experiments performed by NANoREG Task 5.5 and reported in deliverables 5.4, 5.5, 5.6 and 5.7 Deliverable 5.6 comes forward with a scheme providing a first line strategy to collect hazard information at early stages of product development (see figure). Information collected following the proposed scheme may assist product developers in collecting relevant safety information on their potential products, taking into account their final use (route of exposure). The NANoREG WP5 strategy has focused on inhalation and the oral routes as the main exposure routes of entry into the body. The scheme below indicates recommended cell lines per route of exposure, relevant end points and their corresponding assays.

The experiments that have been carried out have resulted in a huge set of well-defined and reliable nanoEHS data that will be of great value in and outside the project.

For more details about NANoREG please visit the official website www.nanoreg.eu.

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