A common European approach to the regulatory testing of nanomaterials

In vitro screening methodology to evaluate toxicity by inhalation
Deliverable 5.4

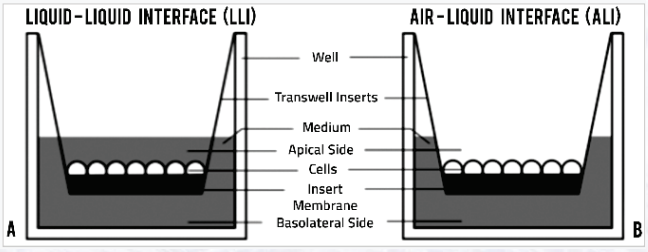
Introduction

Inhalation represents the main route of exposure to nanomaterials (NM). Assessing the risks of this exposure route by performing *in vivo* tests is costly, time consuming and requires the use of animals. *In vitro* tests do not have these disadvantages; however the predictive value of such tests is not yet known.

Task 5.4 of the NANoREG project is aimed at identifying and testing *in vitro* assays that are reliable and best mimic inhalation as route of exposure. The latter will be evaluated in Deliverable 5.05 by linking the results of the *in vitro* experiments to the results of *in vivo* inhalation experiments using the same NMs, carried out in work package 4 (Biokinetics and toxicity testing *in vivo*) of the NANoREG project.

This deliverable (D5.04) presents and compares the results of several *in vitro* tests covering different cell types and endpoints.

Description of Work



Six NANoREG partners evaluated *in vitro* techniques mimicking inhalation exposure by a direct exposure of cells to NMs (air-liquid interface; ALI). Two of them also evaluated the more classical submerged technique alongside the ALI exposure experiments (for more information on submerged cultures the reader is referred to D5.06). Theoretically, the ALI - approach should mimic more closely the typical exposure route of cells in the respiratory tract, namely via air. Partners used the same pulmonary cell models (A549 monolayer, A549 co-culture with THP1 and 3D airway epithelia) and the same four NANoREG core NMs (NM100, NM101, NM212, NM220). Equipment however was not the same.

Main results and evaluation

For each of the involved partners, the deliverable reports on the equipment used, the characterisation of the aerosols, the deposited doses and the cellular responses. Furthermore comparisons are made between partners/equipment, nanomaterials, techniques (ALI versus submerged). Below a selection of the results is presented.

Comparison between different ALI cell models, methodologies and equipment

The six different experimental systems set up to expose cells at the ALI to aerosols of NMs achieved maximum doses of exposure in the order of few µg/cm². Some significant differences were nevertheless noted between the different systems, including the timing of the dose delivery. Except for one partner, the doses deposited were enough to observe significant biological responses (see tables below).

Expression of interleukins IL1β, IL6, IL8. IL10, MCP-1 and TNF-α was studied as biomarkers of inflammation. In general, the low toxicity and few data points collected did not allow for a robust correlation analysis among the different technologies used. Results obtained also highlight the importance of the test system, eg A549 co-cultured with THP-1 being more sensitive than A549 grown in ALI or as monocultures.

	Cytotoxicity						
	A549				A549+THP-1		Lung epithelia
Partner	INERIS	GAIKER	BAuA	TCD	INERIS	KI	GAIKER
NM105	>3				1		

NM101	>3			>0.68	>3		
NM100	>3			>0.68	>3		
NM212	>3	>1.5		>2.72	>3	5	>8
NM220		>1.5	>0.01	0.68			8*

Table: Lowest observed adverse effect levels (LOAEL in µg/cm² for 24 h exposure) for cytotoxicity in cells cultivated at the ALI and exposed to NM aerosols.

	Inflammation				
	A549			A549+THP-1	
Partner	INERIS	BAuA	TCD	INERIS	KI
NM105	>3			1	
NM101	>3		0.68	1	
NM100	>3		0.68	1 to >3	
NM212	>3		2.72	1 to >3	>5
NM220		>0.01	>1.36		

Table: Lowest observed adverse effect levels (LOAEL in µg/cm² for 24 h exposure) for general pro-inflammatory effects in cells cultivated at the ALI and exposed to NM aerosols.

*Toxicity trends observed after 20 days exposure; "no effects" are mentioned in green, "significant effects" in red; Blank cells indicate experiments not carried out by the partners.

Production of reactive oxygen species was also explored by INERIS and BAuA. No increase was observed but for the A549 co-cultured system. An increased ROS production was noted for NM101 but in the co-culture only.

Genotoxicity was performed using two different methodologies on different culturing conditions. Positive genotoxic results were scored for NM100 and NM101 only following the comet protocol. Negative results were however found with the micronucleus assay on cells grown both as monocultures and as ALI. Further information on genotoxicity of NM following *in vitro* protocols can be found in D5.06.

Comparison between ALI and submerged exposures

Regarding the co-culture and considering pro-inflammation results, comparison between ALI and submerged exposures showed that the ALI exposure was a more sensitive model than the submerged one. NM105, 101 and 212 appeared more toxic than NM100 both at the ALI and in submerged conditions *(see table below).*

Table Lowest observed adverse effect levels (LOAEL in µg/cm² for 24 h exposure) determined for pro-inflammatory effects in A549 + THP-1 co-culture for each exposure method used. Cells were exposed at the ALI interface to aerosols of NM or in submerged conditions to suspensions of NM, using NANoREG or INERIS dispersion protocol.

Co-culture	IL-1β			IL-6			IL-8			TNF-α		
	ALI	Subm Nanoreg	Subm INERIS	ALI	Subm Nanoreg	Subm INERIS	ALI	Subm Nanoreg	Subm INERIS	ALI	Subm Nanoreg	Subm INERIS
NM105	1	N.D	10	1	N.D	20	1	N.D	#	1	N.D	20$
NM101	1	10	10	1	#	10$	1	#	#	1	20$	10$
NM100	>3	>20	>20	3	>20	>20	1	#	#	>3	>20	>20
NM212	3	20	>20	3	20	>20	1	>20	>20	>3	>20	>20
NM220	N.D	>20	>20	N.D	#	#	N.D	>20	>20	N.D	>20	>20

"no effects" are mentioned in green, "effects" in red and "potential interactions" are reported in grey. #: Presence of interactions perturbing results, $: Interactions decreasing cytokine levels and perturbing results (uncertain LOAEL), N.D: Non Determined parameter because experiments were not performed

In general, it could be concluded that:

- It was difficult to correlate between ALI methodologies.
- The ALI exposure seems to be a more sensitive model than the submerge one. However, both methodologies provided similar relative ranking of NMs considering their potential toxicity
- It is better to use co-culture cellular models, which proved to be more sensitive under the conditions of the different studies.

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

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