A common European approach to the regulatory testing of nanomaterials

Report on cell type and in vitro-in vivo correlation studies for inhalation toxicity
Deliverable 5.5

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 know.

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 is evaluated in Deliverable 5.05 by linking the results of *in vitro* experiments (work package 5) to the results of *in vivo* inhalation experiments using the same NMs (work package 4: Biokinetics and toxicity testing *in vivo*) of the NANoREG project.

Description of Work

For four sets of data a comparison has been made between the results of in vitro and in vivo assays related to pulmonary toxicity for the same materials. In all the comparisons, mass/cm^2 has been chosen as dose metrics.

Topic	In vivo	In vitro
Pulmonary toxicity of TiO_2 (NM100 and NM101) and CeO_2 (NM212)	Task 4.5.7. Acute and repeated nose-only inhalation toxicity study (D4.15)	Task 5.4. Inhalation toxicity modelling/*in vitro*
Uptake of CeO2	Task 4.4. Pattern of particle distribution in organs	Task 5.6. Develop a rapid high throughput screening methodology
Pulmonary toxicity of nanocellulose	Task 4.7. Acute immunotoxic and genotoxic effects of fibrous nanomaterials	Task 5.5. *In vitro* toxicity assays connected to regulatory questions
immunotoxicity of SiO2 (NM203)	Task 4.5.5. Repeated dose 90 days oral toxicity study	Task 5.5 In vitro toxicity assays connected to regulatory questions

Unfortunately, the long term inhalation study with NM212 could not be included in this deliverable since full set of results will only become available after 2016. Studies included in this deliverable are reported below:

Main results and evaluation

TiO_2 and CeO_2 studies (toxicity studies)

Inflammation LOAEL in µg/cm²		*In vitro* (cytokines IL-1β, IL-6, IL-8, TNF-α)			*In vivo* (cytokines)	*In vivo* (Neutrophils)
		ALI (3h+21h)	Submerged (3h+21h)	Submerged (24h)	IT	IT
TiO_2	NM105	**1**	**3**	**10 - 20**	**0.1**	**0.1**
	NM101	**1**	**3**	**10**	**0.1**	**0.1**
	NM100	**1-3**	> 10	> 20	> 0.1	> 0.1
CeO_2	NM212	**1-3**	**10**	> 20	**0.1**	> 0.1

Below an example of the comparison between *in vivo* and *in vitro* results reported in the deliverable.

Table 1. Lowest observed adverse effect levels (LOAEL in µg/cm² for 24h exposure) determined for inflammation. In vitro A549 + THP-1 co-culture were exposed at the ALI to aerosols of NM or in submerged conditions. In vivo, rats were exposed by intratracheal instillation (IT) to suspensions of NM.

significant effects respective no effect allowing the determination of a LOAEL

Inflammation was the most sensitive endpoint, both *in vitro* and *in vivo*. Focusing on this endpoint, TiO_2 NM were ranked similarly *in vivo* and *in vitro*. CeO_2 appeared more toxic *in vivo* than *in vitro*. In terms of sensitivity the different methodologies can be ranked as follows: *In vivo* (IT) > *in vitro* ALI > *in vitro* submerged in inserts > *in vitro* submerged in plates.

CeO2 inhalation study (uptake study)

The comparison of *in vivo* and *in vitro* uptake results is summarized in the adjacent fig. The mean NP concentration in septum of single alveola was by a factor of 10 lower than in their corresponding in vitro experiments. These were represented by A549 cells cultured as monolayers and exposed to 10µg/ml NM212.The concentration in "hot spots" in alveolar septum was found to be 3 lower than in cultured A549 cells.

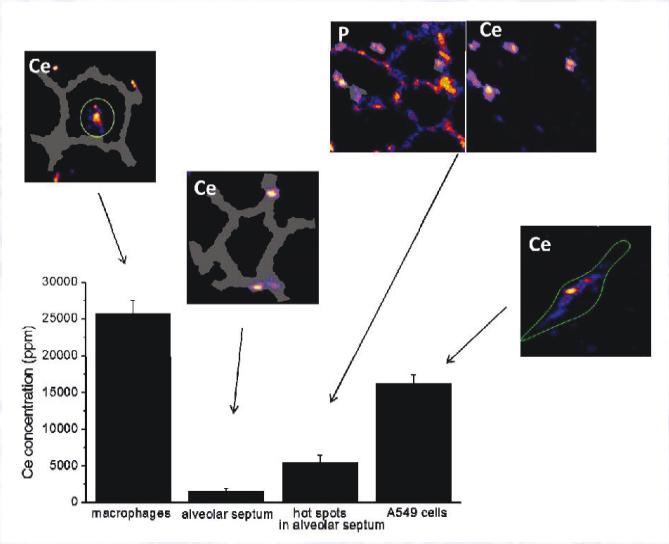

Figure: Uptake, cellular localization and distribution of CeO2 NPs in lung tissue slices from 28 day CeO2 inhalation study of rats as well as in culture A549 cell at applied concentration of CeO2 NPs of 10µg/ml. The cellular concentrations were measured in selected region of interest.

Nanocellulose studies (toxicity studies)

From the experiments with four nanofibrillar cellulose nanomaterial and one bulk size material it is concluded the instillation *in vivo* study is able to yield much more sensitive and refined information on the effects of pulmonary exposure to NFC. The *in vitro* approach underlines the importance of the cellular model used, since different cells may have different sensitivities, selected cellular models should be good representatives of the relevant *in vivo* route of exposure. Under the conditions of the study, in vitro experimentation shows less sensitivity than *in vivo* approach. However, both approaches highlighted the particular toxicity of one of the NFC materials.

The analysis of the two methodologies supports the fact that *in vivo* approach remains at present the reference strategy to characterise NMs immunotoxicity and genotoxicity. *In vitro* approach may provide valuable information regarding the relative ranking of NM if the cell model used is sensitive enough.

Nanosilica study

From the evaluation of the immunotoxicity results obtained in the *in vivo* oral study with NM203 and the *in vivo* study with the same material, it is concluded that *in vivo* study is able to yield much more refined information on the effects of chronic exposure to NMs, whereas *in vitro* approach allows simpler and reproducible evaluation of multiple parameters. The results highlight the need for different experimental strategies (*in vivo* studies and *in vitro* models) to thoroughly characterise NMs immunotoxicity.

Overall conclusions

From sets of experiments described above, the following conclusions can be drawn:

- *In vivo* approach appears to be the most sensitive and exhaustive one to assess absolute pulmonary toxicity of poorly soluble NM.
- *In vitro* approach may provide valuable information regarding relative ranking of NM, provided that the cell model is sensitive enough. In this context it is concluded that monocultures of lung epithelial cells (A549 or BEAS-2B) are poorly sensitive models.
- ALI approach is more sensitive than submerge exposure.
- The development of advanced *in vitro* models, mimicking closer lung physiology and the reality of environmental exposure to assess pulmonary toxicity of low soluble NM should be a priority for the upcoming years.

To improve the *in vitro* predictivity after acute exposure to poorly soluble NMs it is important:

- To assess the real mass of NM deposited on the cell surface in vitro is fundamental
- To use compatible and relevant dose metrics between the *in vivo* and the *in vitro* is critical
- To use more realistic cell models (macrophages ++) and exposure methods

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

This project has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 310584