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TOXICOKINETICS OF MANUFACTURED NANOMATERIALS :

REPORT FROM THE OECD EXPERT MEETING

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TOXICOKINETICS OF MANUFACTURED NANOMATERIALS: REPORT FROM THE OECD EXPERT MEETING

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http://www.oecd.org/chemicalsafety/nanosafety/testing-programme-manufactured-nanomaterials.htm

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The Environment, Health and Safety Division publishes free-of-charge documents in 11 different series: **Testing and Assessment; Good Laboratory Practice and Compliance Monitoring; Pesticides; Biocides; Risk Management; Harmonisation of Regulatory Oversight in Biotechnology; Safety of Novel Foods and Feeds; Chemical Accidents; Pollutant Release and Transfer Registers; Emission Scenario Documents; and Safety of Manufactured Nanomaterials.** More information about the Environment, Health and Safety Programme and EHS publications is available on the OECD's World Wide Web site (www.oecd.org/chemicalsafety/).

This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organisations.

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The Participating Organisations are FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

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FOREWORD

The OECD Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology (the Joint Meeting) held a Special Session on the Potential Implications of Manufactured Nanomaterials for Human Health and Environmental Safety (June 2005). This was the first opportunity for OECD member countries, together with observers and invited experts, to begin to identify human health and environmental safety related aspects of manufactured nanomaterials. The scope of this session was intended to address the chemicals sector.

As a follow-up, the Joint Meeting decided to hold a Workshop on the Safety of Manufactured Nanomaterials in December 2005, in Washington, D.C. The main objective was to determine the "state of the art" for the safety assessment of manufactured nanomaterials with a particular focus on identifying future needs for risk assessment within a regulatory context.

Based on the conclusions and recommendations of the Workshop [ENV/JM/MONO(2006)19] it was recognised as essential to ensure the efficient assessment of manufactured nanomaterials so as to avoid adverse effects from the use of these materials in the short, medium and longer term. With this in mind, the OECD Council established the OECD Working Party on Manufactured Nanomaterials (WPMN) as a subsidiary body of the OECD Chemicals Committee in September 2006. This programme concentrates on human health and environmental safety implications of manufactured nanomaterials (limited mainly to the chemicals sector), and aims to ensure that the approach to hazard, exposure and risk assessment is of a high, science-based, and internationally harmonised standard. This programme promotes international co-operation on the human health and environmental safety of manufactured nanomaterials, and involves the safety testing and risk assessment of manufactured nanomaterials.

As part of its Programme on the Safety of Manufactured Nanomaterials, and in particular work on the testing and assessment of manufactured nanomaterials, OECD initiated a series of expert meetings to discuss the applicability of the OECD Test Guidelines to nanomaterials. With this in mind, the Working Party on Manufactured Nanomaterials agreed to organise an expert meeting to address the toxicokinetics of manufactured nanomaterials. This document presents a report of the discussion and recommendations derived from the workshop.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and Working Party on Chemicals, pesticides and Biotechnology of the OECD.

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Background and Objectives

1. The OECD WPMN Expert Meeting on the Toxicokinetics of Manufactured Nanomaterials was hosted by the Government of Korea and the German Federal Ministry for the Environment, Nature Conservation, Building and Nuclear Safety (BMUB). It was held in Seoul, Korea on 26-28 February 2014. This was one in a series of expert meetings that had been agreed to be held as part of the OECD Programme on Manufactured Nanomaterials. There were 34 participants from twelve OECD delegations.

2. The objectives of the meeting were as follows:

- to discuss key toxicokinetic studies undertaken as part of the OECD Sponsorship Programme on the Testing of Manufactured Nanomaterials[1] (hereafter referred to as the Testing Programme);
- to communicate and discuss challenges in testing;
- to identify methodological gaps and make recommendations for further testing;
- to discuss and make recommendations as to how existing testing methods and respective guidance for toxicokinetics may be improved, complemented and/or more closely integrated with toxicological endpoint testing if required;
- to summarise and discuss the current understanding of the mechanisms and pathways involved in toxicokinetics of nanomaterials, including implications for testing and assessment, and to make recommendations, as appropriate; and
- to discuss and to make recommendations as to how available information on toxicokinetics is being used to enable or enhance risk assessments as well as risk assessment methodology for nanomaterials.

3. The meeting was organised in five sessions and included a keynote lecture from Dr. Wolfgang Kreyling (German Research Center for Environmental Health.

[1] See http://www.oecd.org/chemicalsafety/nanosafety/testing-programme-manufactured-nanomaterials.htm

POTENTIAL PATHWAYS AND MECHANISMS INVOLVED IN NANOMATERIAL BIOKINETICS

Dr. Wolfgang Kreyling

4. This keynote lecture focused on the potential pathways and mechanisms involved in the biokinetics of nanomaterials. The presentation addressed in particular the particle relocation within the lungs as studied using nanomaterials (NM) which, in part, can also agglomerate or aggregate into micron-sized particles (μP).

5. Two series of systematic studies were presented. The first was related to the biokinetics of five nanomaterials (carbon, iridium, titanium dioxide, gold, silver) with the same size (> 20nm) aerosol inhalation. The second introduced the biokinetics of gold nanomaterials (1.4, 2.8, 5, 18, 80, 200nm) and surface modifications (+/- charge, poly(ethylene glycol) (PEG), protein-conjugates) after instillation, injection or ingestion.

6. The lecture showed that nanomaterials relocate from lung epithelium towards lung interstitium. It noted that the inhalation of freshly produced, same-sized 20 nm aerosols of different materials (carbon, iridium, gold, silver, titanium dioxide) cause significant different patterns of biokinetics. It went on to describe how an initial ionic triphenylphosphene surface modification of gold nanomaterials is rapidly replaced by a protein corona within body fluids. Moreover, biokinetics seems to be strongly depending on the physico-chemical parameters of administered nanomaterial and on the route of entry (lungs, gastro-intestinal tract (GIT), and blood). The nanomaterials translocate from the air-blood-barrier (ABB) or the GIT to circulation and are retained in the long-term in secondary organs, tissues and the skeleton.

7. At the same time, the biokinetics of gold nanomaterials differ markedly after lung and GIT administration as opposed to blood administration; hence, gold nanomaterials crossing the ABB or the GIT epithelium cause major changes to the protein corona when compared to blood administration. The translocation of gold nanomaterials across the ABB has been defined to be about 10-fold higher than the absorption of gold nanomaterials across GIT; yet the biokinetics patterns are rather similar suggesting similar protein coronas.

8. The quantitative biokinetics of the NMs within the entire organism is required as an important building block of subsequent toxicological studies and, hence, a rational risk assessment. From the small fractions accumulated in secondary organs after short-term exposure no adverse health effects are likely. However, NM may trigger mediators in the primary organ (e.g. lungs) released to blood; these mediators may well initiate adverse health effects in the cardio-vascular system and elsewhere. During chronic exposure either to lungs or to gut, NM concentrations in secondary organs may accumulate high enough to trigger adverse health effects; unfortunately very few long-term exposure studies have been carried out to date.

TOXICOKINETICS STUDIES AND DATA FROM THE OECD TESTING PROGRAMME

Chair: Agnieszka Kinsner Ovaskainen (EC, JRC), Rapporteur: Ghita Falck (EC, ECHA)

9. The objective of this session was to discuss results of key toxicokinetic studies performed in the OECD Testing Programme, complemented by biokinetic data from the EU-Nanogenotox project, RIVM and BfR work, and to discuss challenges specific for the testing of nanomaterials. Furthermore, the session aimed to identify methodological gaps and to suggest recommendations for further testing. In addition to key findings, attention was given to the study designs used, the experience gained, as well as the technical challenges that were encountered.

10. The session included a number of presentations addressing the toxicokinetics of fullerenes, MWCNTs, TiO_2, SiO_2, ZnO, silver and gold nanoparticles. It should be noticed that several nanomaterials were considered for each chemistry representing different sizes and different surface treatments.

11. The performed studies were done via oral, inhalation, instillation and intravenous administration according to the OECD guidelines: TGs 403, 407, 408, 412, 413, 417 and 420. It was noted that many of the studies represented combined toxicity and toxicokinetics studies, rather than those dedicated to kinetic studies. The following paragraphs provide a summary of the main observations for the different materials studied.

12. *Fullerenes*: Different formulations containing C60-fullerenes were administered by gavage to rats. Generally, more than 85% of the dose were recovered with faeces. In the absence of mass balance measurements, the exact degree of oral absorption could not be determined, but it was clearly variable between the types of fullerenes / formulations administered. Very low (typically 0.1-1.5 µg/g wet weight) or non-detectable concentrations were reported in brain, intestinal lymph nodes and blood. When injected into the tail vein, fullerene was rapidly cleared from blood circulation and localised in liver, lung, spleen and kidney. When instilled into lungs, >90% of the fullerene was estimated to be cleared rapidly. Part of the fullerene was retained in the cytoplasm of alveolar macrophages and alveolar epithelial cells for an extended period.

13. *MWCNTs*: Single exposure studies are to be used only to validate kinetics and the morphology of the retained MWCNTs in the target cells. It was emphasised that improved techniques are needed for the determination of the apparent density of particles in inhalation chambers. After acute inhalation exposure (4-6 h), the deposition of MWCNTs (L/D: 7 + 3 µm / 35 + 12 nm) in the lungs was about 10% of the administered dose. Within one day, most of the MWCNTs were engulfed by alveolar macrophages and cleared from the pleural space to the lymph system. It was noted that it is critical to design studies that respect the macrophage overload doses (i.e. not to use doses high enough to cause macrophage overload). After intratracheal spraying, long MWCNTs (7-8 µm) were found on both sides of the pleural space, while short MWCNTs (< 3µm) were cleared by pleural macrophages. Mesothelial proliferation in the pleura was induced by long MWCNTs only (long term process). There was an increase of inflammation markers in the pleural lavage fluid induced by both MWCNTs, and it was more pronounced after exposure to the longer MWCNTs. In general, long MWCNTs have more potential to cause inflammation or proliferation as with asbestos-like pleural disease than short MWCNTs. Problems were encountered with the dosing and quantification of MWCNTs. One of the methods proposed to reliably analyse the carbon originating with and released from MWCNTs in the lung was the total carbon method. There were questions raised as to whether animal models are adequate to reflect human exposure of asbestos-like materials. After oral administration MWCNTs low if any detectable amounts were absorbed from the intestinal lumen. To nevertheless address biodistribution and –persistance, i.v. studies were performed, showing distribution

mainly to liver, spleen and lung. Major differences in translocation, distribution and clearance between the investigated MWCNT nanomaterials (NM400, NM401, NM401 and NRCWE-006) were noted following repeated i.v. injection. While forNM-400, a decrease was noted between day 6 and day 90 in liver and spleen but not in lung, there was minimal decrease from day 6 to day 90 for NM-401 and no decrease until day 90 for NM-402 and NRCWE-006.

14. *TiO_2*: Various TiO_2 NMs were used in the studies presented. The studies were performed via the oral, intravenous (i.v.) and inhalation routes. TiO_2 NMs were rapidly cleared from the blood (5-20 min) after i.v. administration. Accumulation in liver, lung, kidney and spleen up to 60-80% (liver) and 1-3% (lung and spleen) of the administered dose following i.v. injection was found. The highest accumulation of the TiO_2 NMs was detected in the liver and spleen. A remarkable difference for clearance from examined organs over 90 day was noted between one TiO2 nanomaterial (rutile-anatase mixture; NM-105) as compared to other TiO_2 materials. Even for NM.105, which was cleared faster, considerable 5-10% of the dose were recovered from the four examined tissues at day 90 after administration, compared to approx.. 40% at day 6. This suggests extremely slow clearance, indicating a potential for accumulation. Uptake was almost identical in male and female rats. Low absorption of Ti from the GI tract was noted. Only in incidental cases (8 out of 50 determinations) TiO_2 translocated from the GI tract in a very low amount. Only 0.002 % translocated to the mesenteric lymph nodes. Differences between different TiO_2 NMs were minimal. In a 28 days inhalation study with post recovery phase in which three different TiO_2 (NM-103, NM-104, NM-105) were administered, the retention half-times were between 1 – 1.3 years. Most of the particles were localised in the lungs. The concentrations in liver and brain were below the limit of detection. When an identification of the respiratory cell types responsible for uptake of these particles was performed, the intra-alveolar macrophages were the most prominent compartment where particles were detected. No significant differences were observed in the kinetic behaviour and in toxicological response when comparing the three TiO_2 NMs.

15. *SiO_2*: Tissue distribution, elimination and oral absorption of synthetic amorphous silica were studied in rats using two well characterised particles (NM-200: precipitated with primary particle size 14-23 nm and NM-203: pyrogenic with primary particle size 13-19 nm). Deposition in tissues after repeated oral administration was low but measurable in liver and spleen at both day 6 and day 14. Particle and gender-related differences were found. After 90 days the Si levels had declined but were still measurable in liver. After intravenous administration, Si peaked in blood but was then rapidly eliminated. At day 90 the Si content in liver and spleen was still higher than in the controls. Another study with two types of colloidal silica were (20 / 100 nm, E&B Nanotech, Korea) being orally administered (single dose) was analysed with regard to technical challenges and lessons learned. In particular, problems were encountered with unsatisfactory mass balances (recoveries), lack of data on residual carcass and the use of an excessive dose not relevant to real world exposure but based on the high dose used in toxicity studies. In the inhalation studies the amorphous SiO_2 (NM-200) was eliminated without detectable translocation to remote organs and no significant amount detected besides in the lungs (in intra-alveolar macrophages).

16. *ZnO*: *In vitro* and *in vivo* studies (14 days, 90 days inhalation studies, acute toxicity test (route and dermal penetration) were performed with two nano-sized ZnO and one micron-sized ZnO. One of the ZnO nanomaterials was coated which reduced its solubility, but the other not. The deposition of ZnO was higher for one of the nanosized ZnO in the lungs. ZnO particles were not detectable by TEM in the other tissues examined.

17. *Ag*: Three studies involving silver nanoparticles were presented during the session. Ag-nanoparticles of different physicochemical properties, e.g. average primary particle sizes between 10 and 60 nm were discussed with the aim to identify the organs at risk for adverse effects and to determine the silver content in blood and organs. It was concluded that the particulate nature of nanomaterials influences the toxicokinetics. The toxicokinetics seems to be dependent on size, shape, material, amongst other

parameters. After intravenous administration of silver nanoparticles, a distribution to and accumulation in liver and spleen was detected, with the indication that there may be a risk of bioaccumulation in chronic exposure settings. The particulate nature appears to influence the absorption and distribution of silver nanoparticles. A difference in absorption and distribution between sizes was observed. A 7-day and 28-day oral study was also conducted. The 28-day study showed that silver is bioavailable from the GI tract. In another 28-day oral study biopersistence in the brain and testis was detected. In a 90-day study silver was distributed to all organs with a gender-specific accumulation pattern for kidneys. Finally, silver nanoparticles from JRC repository (NM-300), certified by the Federal Institute for Materials Research and Testing (BAM, Berlin) was investigated *in vitro* in a human intestine colon carcinoma cell line (Caco-2) as a cell barrier model in a Transwell system for its translocation potential. A dispersion of NMs prepared sequentially in human gastrointestinal digestion fluids was used containing either artificial saliva, or stomach juice, or gut digestion fluid with relevant enzymes necessary for nutrient digestion according to DIN 19738 (2004). Linda Böhmert et al (2014) analytically monitored the digestion of these reference silver nanoparticles and their toxicity in intestinal cells (Nanotoxicology 8(6): 631-642). During digestion particles size decreases and retain their toxicity to cells *in vitro*.

18. *Au*: A 28-day intravenous injection study showed that gold nanoparticles were distributed to liver, spleen, kidneys and lung. Furthermore, gold was found to be biopersistent in kidney, spleen and lung after the injection studies. The 90-day study showed a gender difference for bio distribution to kidneys. The major organs to be affected by accumulation in the 90-day study were lung and kidneys.

Discussion and conclusions

19. It was noted that TG417 was not used but instead there was the inclusion of kinetic endpoints in subacute or subchronic studies. TG417 (including the update that became available in 2010) was designed primarily for chemicals for which the kinetics is governed mainly by diffusion/perfusion and metabolic processes, rather than particulates which behave fundamentally diferent with respect to absorption, distribution and clearance. This applies also to the time-frames recommended for exposure and post-exposure observation time (at a maximum of 7 days). Also there are no considerations with respect to test item preparation and other relevant aspects for the inhalation route. Finally, relatively small changes in the exposure situation can have significant impact on the kinetic behavior in particular for inhalation studies. At the current time, this is not sufficiently addressed.

20. It was generally considered that the analysis of tissue and excreta should discriminate between particulate and dissolved material. However, it was noted that there are currently no sufficiently developed and validated methods available. Thus, further development of this area was recommended.

21. The choice of (an) analytical method(s) will depend on the material under investigation and may include TEM-EDX, TEM-EELS[2], while also widefield optical methods such as Cytoviva, PLM[3] and DFM[4] may be particular suitable if concentrations are low. Chromatographic and filtration and other techniques were also mentioned. Single particle Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and Field-Flow Fractionation (FFF) with optical Ultra Violet (UV) Multi Angle Light Scattering (MALS) detection may be suitable for quantification of inorganic particles. It was mentioned, that the various methods will produce results that can have different metrics, e.g. particle numbers vs. mass concentrations, creating further issues that need to be addressed.

[2] TEM: transmission electron microscopy; EDX: Energy-dispersive X-ray spectroscopy; EELS: Electron energy loss spectroscopy.

[3] PLM: polarised light microscopy

[4] DFM: dark field microscopy

22. It was also noted that drawing a mass balance for NMs poses technical challenges that are presently very difficult to solve with available methodologies and analytical techniques. There is still a knowledge gap on the biotransformations that NMs undergo *in vivo* (such as degradation/dissolution and all intermediate states between particulate and dissolved materials). Furthermore, a wide variability is to be expected even for NMs having the same chemical composition depending on, amongst other things, size, aggregation behaviour and surface properties. This means that an ad hoc study could be necessary in order to draw a satisfactory mass balance for a specific NM, which could be more complex and time consuming than the toxicokinetic study itself. It has to be appreciated that total recoveries of administered test substance in the order of > 90 % are incomparably more difficult to obtain for NMs than for traditional chemicals.

23. If a single dose is used, it should be non-toxic but high enough to allow merely the detection of the NM and related dissolved materials in tissues and excreta and thus its tissue distribution. Finally, while a single dose can be regarded sufficient, two, or more, doses should be considered. This is because information gathered from at least two dose groups aid in dose setting in other toxicity studies. Furthermore, dose-response assessment for systemic effects can be complicated for NMs by the lower bioavailability of dispersions prepared at higher concentrations due to agglomeration/aggregation. An example of this has been seen with silica (lower tissue deposition at higher oral doses).

MODELLING TOXICOKINETIC BEHAVIOUR OF NANOMATERIALS FOR HAZARD & RISK ASSESSMENT

Chair: Wolfgang Kreyling (DE); Rapporteur: Jos Bessems (EC, JRC)

24. This session addressed the modelling of the toxicokinetic behaviour started with a presentation by Jos Bessems (European Commission, Joint Research Centre) *PBK modelling in Human Health Risk Assessment*. The Physiologically-Based Pharmacokinetic (PBK) modelling approach is used more and more in the soluble (bulk) chemicals arena in which kinetics is driven mainly by the diffusional passage (permeation) of the protecting outer membrane (skin, gut or lung -air-blood-barrier) to circulating blood, followed by diffusion into perfused organs and tissues, metabolism and finally excretion. The behaviour of NMs in mammals appears to be quite different. Their uptake and disposition is not driven by diffusion but by active local uptake by alveolar macrophages followed by local clearance towards the larynx (at least upon inhalation) and, in addition, by epithelial cells towards lung tissue and blood vessels; and once systemically available by active clearance from the blood, again mainly by macrophage-type cells. So far, organ disposition has been modelled only by a fitting term, meaning without a mechanistic term. There seems to be some kind of sequestering in the macrophage type of cells. Whole body clearance appears to be very slow. Currently, main use of PBK models for NMs is hypothesis testing: How fast is the translocation to secondary organs? What is the mechanism and what are the accompanying descriptors for the likely one-way direction uptake into secondary organs (such as rate of uptake by macrophages)? The main message was that PBPK modelling is not yet ready to be used wide-scale for various extrapolations in Human Health Risk Assessment (HHRA).

25. The second lecture was given by Claude Edmond from University of Montreal (Quebec, Canada) A physiologically based pharmacokinetic model development to study the fate and behaviour of manufactured nanomaterials in rodents. He advocated trying to reproduce at least the rate limiting steps in the various steps along the continuum. The complexity of the model depends on the degree of description. There is no final model and the use, purpose and hypothesis with respect to the relevant process determines the required complexity. Any model can be continuously improved with knowledge improvement as necessary for the use purpose.

26. Claude Emond reiterated the message from the first speaker that PBPK modelling as a tool could certainly be used for various extrapolations but we are at the beginning of kinetic modelling for NP. Complexity of PBK modelling of nanoparticles compared to PBK modelling of 'classical' readily soluble chemicals concerns many aspects such as physical property changes, local uptake by alveolar macrophages versus epithelial cells, systemic translocation or absorption, protein corona formation, cellular recognition, uptake by systemic phagocytosing cells and finally, excretion. The uncertainty analysis of their recently published PBK model[5] revealed that the model outcome was most sensitive to three parameters: uptake capacity as well as maximum uptake rate of phagocytosing cells in organs and the partitioning coefficient between blood and tissues.

27. The third lecture was by Jürgen Pauluhn (Bayer AG / BIAC) Hypothesis-based generic design of inhalation toxicity studies for nano- and microscaled biopersistent poorly soluble particles'. He argued that single inhalation studies are clearly preferred over single instillation approach due mainly to the non-physiological exposure and subsequent reactions that the latter might give. Results indicated that for the

[5] Li et al, Physiologically based pharmacokinetic modelling of polyethylene glycol-coated polyacrylamide nanoparticles, Nanotoxicology, 2014 Aug;8 Suppl 1:128-37. doi: 10.3109/17435390.2013.863406. Epub 2014 Jan 6

investigated Baytube 2µm agglomerated particles, the alveolar macrophages (AM) are the accumulating compartment, not the epithelium. So for agglomerated particles there exists presystemic (local) accumulation. Other participants commented that this may not be necessarily the case for particles in the nano range.

28. It was agreed during the short discussion that studies on biopersistent, low-toxicity, nano-structured materials require thorough biokinetics measurements and a long enough study period of e.g. 13 weeks followed by at least 13 weeks of recovery to better understand the effects possibly seen. But there was disagreement on the assumption that nano-structured particles are solely phagocytised by AM. It is a genuine feature of nanomaterials to be phagocytised by epithelial type I cells as well. The latter relates to the fact that the number of depositing nanomaterials are too high for AM and there is only very limited recruitment of PMN[6] since the AM do not send inflammatory and chemokinetic mediators as there is no volumetric overload occurring in any of the AM. Hence, epithelial cell endocytosis covering the entire epithelium is competing against AM phagocytosis which is making up only 1% of the epithelial cell population.

29. Finally, Carsten Kneuer (BfR, Germany) presented *Comparing predictions of Multiple Path Particle Dosimetry Model (MPPD)[7] to measured data from the OECD Sponsorship Programme*. For TiO_2 NMs the MPPD provided a reasonable prediction of deposition in short-term exposure, but appeared to be less predictive for subchronic exposure. The reason might be that local lung clearance is overestimated by the MPPD model leading to an underestimation of deposition. For nano Ag the overall findings were similar albeit now clearance was apparently underestimated (and deposition overestimated). It was argued that this occurs since the MPPD model does not account for any dissolution of the AgNP which probably will be the most prominent clearance pathway of AgNP: conversion -into non-particulate forms of Ag as well as some formation of very small clusters of e.g. Ag-chloride. For nano Au the predictions were overall not satisfying, like with TiO_2, clearance may be over predicted. The overall conclusion was that a reasonable prediction of deposition after short-term exposure was observed but the prediction of lung burden following repeated exposure to nanomaterials, MPPD should be used with great care due to issues with respect to clearance, solubility and possibly regional deposition of small heavy material.

Discussion and conclusions

30. Participants discussed non-testing approaches and modelling based hypothesis formulation. It was noted that solubility appears to be an important consideration for toxicokinetics of nanomaterials. The issue of continuous dissolution for metal particles was raised. Thus, it could be useful to develop a guidance model that considers dissolution in biological media and to learn how this influences the disposition, for example, at various pH conditions. An example given was the case of silver, which is extremely reactive with various ligands. Furthermore, dissolution and formation of Ag+ and formation subsequently of Ag salts and clusters need to be taken into account when analysing Ag nanoparticles. Participants agreed that a harmonized approach is necessary for all nanomaterials since solubility is an important component. It was suggested that AgCl, ZnO and soluble Zn-salts should be used as controls. $Zn_3(PO4)_2$ may directly form nanomaterials.

31. It was also noted that guidance is needed on how to measure the metal speciation. Dissolution kinetics and subsequent reactions of the formed ions were seen as important. However, caution was raised with respect to the approach used by the USA-CAN Regulatory Cooperation Council (RCC). It uses solubility in water for decision making as default, while in practice it is not water but physiological salt

[6] PMN: Polymorphonuclear leukocytes

[7] MPPD: Multiple-Path Particle Dosimetry

solutions, often containing some proteins as well (such as in lung mucus), that that may constitute a more realistic exposure condition.

32. Participants noted that the intended use of *dissolution* (for regulatory purposes) would affect the requirements with respect to precision and other aspects of reliability. For example, using dissolution measurements for categorisation of materials could be suitable to categorise materials as soluble and potentially soluble but certainly not (yet) for quantitative risk assessment. Still, it was argued that even preliminary (semi-quantitative) data on e.g. the dissolution rate constants could be informative for a first-tier risk assessment.

Q1: If highly soluble, would it no longer have to be considered as NM?

33. Dissolved metal species could be relevant for Ag, ZnO and SiO_2, but not for TiO_2. The dissolution is important with respect to persistence and proposals for read across between bulk ZnO and ZnO nanoparticles. If dissolution kinetics and subsequent/parallel toxicokinetic behaviour is the same, this would support read across.

34. Regarding the issue of metabolism and transformation of silver nanoparticles, it is important to understand the behaviour of the bulk Ag and Ag nanomaterial. The Ag nanomaterial may have behaviours and effects different from those of the Ag-bulk counterparts. Because of the high costs of *in vivo* testing it is important to consider dissolution kinetics, starting in *in vitro*. A project group (led by South-Africa) within ISO TC 229 WG 3 (Working Group on Health, Environment and Safety) is working on this issue.

Q2: What is the role of alveolar macrophages phagocytosis for NM in comparison to microscale particles?

35. When there is deposition of nanosized particles on the epithelium, then macrophage clearance is less likely to be important than for larger (>100 nm) agglomerates, and translocation into and through the epithelium may be more relevant for nanosized particles.

36. NM may agglomerate significantly, depending on its physicochemical properties and concentration. Exposure to nanomaterials as such does exist, although exposure to microscale particles seems to be more important because of this agglomeration behaviour of NMs. Separate toxicity experiments should be performed with either with agglomerates or with NP, for examples in cases the MPPD[8] model does not work.

37. It was reasoned by the participants, that studies on NMs would be relevant at low human exposure concentrations. At higher concentrations (e.g. in occupational setting) NMs are expected to show a high tendency to agglomerate whereas at lower human or environmental exposure concentrations this may not be the case. It is relevant to examine the complete life cycle and the exposure characteristics in all stages, including potential for dispersion and de-agglomeration. For TiO_2 there was agreement that inhalation exposure generally would occur to agglomerates above 100 nm, but exposure specialists should be consulted.

Q3: From a risk assessment point of view, is it important to know whether the exposure is related to agglomerates or to the nanoform?

38. For a risk assessor, the important question is to know how the rate of de-agglomeration of agglomerates compares to uptake rate by macrophages. It has been suggested that the role of agglomerate is an interesting point, when considering phagocytosis of particles of 500 nm and above. Below 500 nm

[8] MPPD: Multiple-Path Particle Dosimetry

there is a competition with endocytosis by epithelial cells. Importantly, the intracellular environment is really different in macrophages compared to epithelial cells. Sometimes, attempts to create NM reveal no single NM or agglomerates below 100 nm. Studies are done to compare different techniques for generating an atmosphere containing nanomaterials, which appears to be a challenge as they often result in only a very small number of particles below 100 nm.

39. On the other hand, biologists and toxicologist might not perceive thermodynamics sufficiently (Gibbs free energy). It costs energy to obtain a corona of proteins. Furthermore, in biological systems, energy is not available to break agglomerate bindings. However, it was mentioned that the relatively small agglomerates would de-agglomerate quite easily in the lungs. It has been mentioned that early studies on e.g. carbon black showed that there is translocation into the surface area of the lung.

Q4: How does macrophage clearance compared to other clearance? Is it different?

40. Many experiences exist to measure the exposure (e.g. CNTs dispersed fibre exposure). Alveolar macrophages (AM) do recognise larger micro sized particles more easily, but this remains relative. Probably also many real NM (<100 nm) do reach the macrophages.

41. It has been noted that only 20% is taken up by Alveolar Macrophages (AM). For micro sized particles this percentage is much higher (mass based). If this is based on mass, then still 20 % can be a very large number of particles.

42. Based on the Session 1 discussion regarding Au and Ag, irrespective single particle or agglomerate inhaled, the particles in the tissues look similar.

43. Finally, the importance to properly understand translocation to epithelium when understanding overload, has been underlined. If so, then the role of macrophages is absolutely critical.

44. All in all, the discussion on the (relative) role of pulmonary macrophages made it clear that there is no consensus in this issue.

Q5: Is it possible for the NM to enter into the cell or not?

45. On the opposite, many papers show that non-phagocytic cells can take up nanomaterials also (endocytosis). When *in vitro* 'upside down' culturing is applied significant uptake in the epithelial cells is observed.

46. It has been argued that considering alveolar macrophages uptake of nanomaterials and the ciliary transport to the upper airways and potential swallowing, subsequent 'oral uptake' should be considered and distinguished as well.

USE OF TOXICOKINETIC INFORMATION IN RISK ASSESSMENT

Co-Chairs: Carsten Kneuer (Germany), Il Je Yu (Korea)

47. This session started with a review on the use of toxicokinetic information in the risk assessment of NMs. The presentation made reference to the OECD work on risk assessment of manufactured anomaterials [9]. Examples were provided on how kinetic information may be used to compensate for gaps elsewhere in the risk characterisation. The concept of "Lack of Bioavailability or Toxicity" was described, which means that circumstances may exist where exposure characterisation is not possible or practical, but where there is considerable weight-of-evidence showing a lack of toxicity, including in chronic toxicity testing which is supported with ADME evidence indicating no concerns regarding biopersistence and bioaccumulation. It was noted that when toxicokinetics of a nanomaterial is known to depend on certain physicochemical parameters, these parameters should be considered in the substance identification of the nanomaterial. This was linked to lifecycle considerations, that is, the toxicokinetics of a NM may change as the result of "modifications". To judge the relevance of TK studies for risk assessment, a better understanding must be developed on how different exposure conditions in testing and real-life are related. Improving predictive (computational) models may be helpful in this context, but also for other purposes. It was concluded that toxicokinetic information may be highly relevant for hazard and risk assessment of nanomaterials, but more specific guidance on testing and interpretation / use is needed to put concepts into practice.

48. A presentation was provided by Karin Wiench (BIAC) on the case study, describing how dermal absorption studies of a range of nanomaterials have been employed for decision making in risk assessment. The *in vitro* dermal penetration study described in the OECD test guideline no. 428 has been used to determine potential uptake of nanomaterials used as sun screens in cosmetic formulations. Early studies demonstrated that there was no detectable permeation of titanium dioxide and zinc oxide nanomaterial in cosmetics through intact skin with this method. Modifications to the test method were developed which were related to the analytical techniques for quantification of the material in skin and receptor medium. The risk for the nanomaterial applications was assessed as minimal since no contact with living parts of the skin could be foreseen, based on the test results. Clearly, this can be different for other nanomaterials and formulations and this is why testing is necessary. The OECD test guideline no. 428 offers an accepted *in vitro* method using human tissue to address this early step of the assessment of the risk associated with the dermal exposure to nanomaterials. The discussion on the safety of nanomaterials used as sunscreens in cosmetics extended, however, beyond the standard testing used so far for molecules; debating also the penetration of compromised skin. This was addressed by a study in sunburned skin. It demonstrated that the UVB[10]-damage enhanced the nanomaterial penetration from sunscreen formulations but no transdermal absorption was detected with the current method.

49. In another presentation, Wim de Jong (Netherlands) provided an analysis of general tendencies in the toxicokinetic behaviour of nanomaterials and concluded on the consequences for risk assessment. When assessing particulate material, the kinetic behaviour of the particle as well as of any dissolved material has to be taken into consideration. Particulate material always presents as a non-uniform mix of particles (for example, of different sizes, shapes), unlike soluble substances that can be considered as the sum of identical molecules behaving identically. Agglomeration of particles in the GI-tract or the aerosol can be source of lack of correlation between dose and accumulation/response. Compared to soluble

[9] OECD (2013) Prioritisation of Important Issues on Risk Assessment of Manufactured Nanomaterials [ENV/JM/MONO(2013)18].

[10] UVB: Ultraviolet B (280–315 nm)

chemicals, the expected uptake of nanoparticles is very low (<<10%), but can be higher for dissolved material from the nanomaterials. Overall, the kinetics of nanomaterials is governed by other processes than for molecules: Transport of particles across barriers is, unlike for most molecules, not based on partitioning, which is gradient-driven, but on active (energy-consuming) transcellular transport. Particles are not expected to be metabolised. Distribution is through active processes of the mononuclear phagocyte system (MPS) rather than diffusion. It maybe carrier-mediated and be affected by corona formation, amongst other things. In any case, the kinetics of NMs cannot be extrapolated from the dissolved form. This was exemplified for silver nanoparticles and silver nitrate. It was concluded that to date, the toxicokinetic behaviour of particles is complex and hardly understood. Further research is needed to improve this understanding and perform better risk assessment of NM. The latter is complicated as the result of low absorption/bioavailability and high accumulation once systemically available, at the same time. Also, some changes would be necessary in testing. Rather than blood (plasma) concentrations, tissue levels including liver, spleen and lung (for inhalation) need to be tested. Accumulation in tissues should deserve attention but cannot be addressed in typical single-dose or short term toxicokinetic studies. Knowledge of the ADME of nanomaterials is also needed for correct interpretation of toxicity testing. For example, if the organ was not reached, the negative outcome of an *in vivo* genotoxicity test will be irrelevant for the assessment of hazard to another organ or the whole organism. Finally, it was proposed, that risk assessment should preferably be based on internal dose (concentration, internal amount of nanomaterials), i.e. tissue burden, rather than external exposure dose to reflect low but variable bioavailability and mostly high accumulation potential.

Discussion and conclusions

50. Participants discussed how toxicokinetic information can inform risk assessment and management in different settings. Specifically, the type and extent of information required was discussed, also in relation to the need for development or adjustment of test guidelines and guidance.

51. It was also noted that more data should be generated to improve our mechanistic understanding of the biokinetics of nanomaterials and on how potential accumulation of the low percentage that becomes systemically available affects long-term risk especially with repeated/chronic exposures.

52. The following specific questions were discussed by the expert meeting participants:

Q1: TG417 recommends sampling of liver, fat, GI tract, kidney, spleen, whole blood, residual carcass, target organ tissues and any other tissues (e.g., thyroid, erythrocytes, reproductive organs, skin, eye (particularly in pigmented animals) of potential significance in the toxicological evaluation of the test substance. Analysis of additional tissues at the same time points should be considered to maximize utilization of animals and in the event that target organ toxicity is observed in sub-chronic or chronic toxicity studies. Do we have the same data need for NM from the RA perspective? Do we need other/additional/less information?

53. Participants agreed that the selection of tissues to be sampled and analysed should allow calculation of internal dose, identification of potential target organs and target organ burden. In addition, the selection of samples should allow the estimation of clearance, that is, by including excreta.

54. The selection of organs to be evaluated in toxicokinetic studies for NM should include those along the expected pathway(s) for the particular route of exposure such as local lymph nodes, blood and kidneys; the application site; tissues of the mononuclear phagocyte system (MPS) such as liver and spleen; and tissues of interest / concern, namely olfactory bulb (for inhalation), brain, bone marrow. Heart was not considered an organ of concern as cardiovascular effects were thought to be indirect. For inhalation,

broncho-alveolar lavage may be sampled separately from the lung. Pleura should be considered for fibrous materials.

55. It was noted that accumulation may occur in tissues like testis and fat depending on the material. This may be linked to dissolution of the nanomaterial. Relevant information on the appropriate selection of (other) organs may be provided by toxicokinetic studies on other forms of the material or from toxicity studies.

56. The remaining carcass should be included in order to allow for determination of total internal dose (mass balance) and overall recovery.

57. For some organs, the sample size may be too small and/or the method not of sufficient sensitivity. Determination of mass balance will mostly not be very informative due to the low overall fraction absorbed. Sufficient analytical recovery from tissue matrices should be demonstrated, however.

Q2: It was suggested that when there is initial concern (high availability / low clearance), more TK information may be needed. Can we envisage a two-step process, building on a pilot study (as also in TG417 for study design)?

58. Participants agreed that a pilot study may be helpful to define the design of the main kinetic study. However, it was also noted that toxicokinetic behaviour can be affected strongly by the exposure conditions and in an unpredictable manner given the current state of knowledge.

Q3: It was stated that when risk assessment is based on external dose and extrapolation to realistic lower doses, this (non-linearity) may result in an underestimation of risk. Do we agree? What are implications for testing? What are implications for assessment?

59. As the toxicokinetics of nanomaterials can be heavily and (currently) unpredictably influenced by exposure conditions, it should be investigated under the conditions as in the relevant toxicity tests. This may be achieved by inclusion of satellite groups. At the same time, however, information obtained in particular at high doses – such as those typically tested in animals - can be irrelevant to human exposure situations examined in the risk assessment.

60. As concern arises in particular from the low clearance and subsequent accumulation of the small fractions absorbed following individual exposures, kinetics may be more appropriately addressed with repeated exposure scenarios. This may also resolve limitations arising from insufficient analytical sensitivity, which may be too low to measure translocation with single exposure.

Q4: If you want to provide a guidance document that supports risk assessment for nanomaterials, which other considerations on kinetics would you want to put in there?

61. While for conventional chemicals, information on toxicokinetics of the substance is used for route-to-route extrapolation, there is currently no approach how (and whether) this would be feasible for nanomaterials.

62. Dose-metrics require further consideration. For example, agglomeration at higher concentration in inhaled aerosols was considered by the participants to reduce density, thus increasing volumetric load of macrophages (relative to mass). Thus, there are additional sources for non-linear kinetics of nanomaterials compared to conventional chemicals.

63. In addition, information on toxicokinetics, including preliminary data, can be used to judge the relevance of doses in toxicity testing and to draw attention to potential target organs of toxicity.

Q5: How can the toxicokinetics of nanomaterials and their bulk counterparts be used to inform the need for a harmonized set of test guidelines for ultimately deriving human health and environmental standards?

64. There was agreement between the participants that toxicokinetic information can be particularly useful to decide on the possibility of read-across of toxicity data between two nanomaterials of the same chemical composition or the nanomaterial and its bulk counterpart. Similarly, toxicokinetic information would be useful to support bridging within groups/categories of nanomaterials.

TESTING METHODS FOR DERMAL ABSORPTION ASSESSMENT

Co-chairs: Mario Goetz (Germany) and Karin Wiench (BIAC)

Objectives and process

65. Testing protocols (TG427, TG428 and alternative protocols including GD 156) for dermal absorption testing shall be discussed with regard to strengths, limitations and reliability. Recommendations for further development of the protocols may be made. A list of specific questions was prepared and collected from participants to guide the discussion.

Introductory Statement on Dermal absorption testing using OECD TGs

Karin Wiench (BASF, BIAC)

66. Dr. Wiench provided an introductory lecture on dermal penetration studies using *in vivo* (TG427) and *in vitro* (TG428) methods and reasoned on the advantages and drawbacks of currently used experimental approaches. In 2004 the GD 28 was published specifying experimental needs to circumvent pitfalls in skin absorption testing. Since then further Guidance Notes on Dermal Absorption (No. 156) were released by OECD in 2011. It was highlighted that the effects on the permeability of chemicals through skin will depend on the species, the dosing regimen (single or multiple treatment), the skin thickness, the sample type (excised vs. reconstructed), the composition of the donor fluid, the concentration in the donor fluid and the composition of the receptor fluid, and the post exposure time. In addition, the interaction of the nanomaterial with the skin can be influenced by its (physico)chemical properties, as was exemplified by a comparison of the ZnO forms Z-cote (NM110) and Z-cote HP1 (NM111).

Lessons from the Australian sunscreen study with human volunteers

Maxine McCall (CSIRO, Sydney, Australia)

67. Dr. McCall presented lessons learned from the Australian sunscreen study with 20 adult humans of skin types I-IV. It was highlighted that Zn from ZnO particles (enrichment 99.9 % ^{68}Zn) in sunscreen (20% w/w oil water formulation) applied to skin was systemically absorbed under conditions of use. There was a minimal absorption of bulk and nano-ZnO with crystallite sizes of on average 19 and 110 nm, respectively, whereas no significant differences were observed between males with bulk and nano-ZnO and females with the bulk ZnO. Higher ^{68}Zn levels were measured in females who received the NM sunscreen formulation (ICP-MS with simultaneous collection of various Zn-isotopes), which only represent about 0.1 % of normal Zn levels naturally present in the body.

68. The levels of ^{68}Zn in blood increase with the number of sunscreen applications and continue to increase post administration. Since there is a lag time of absorption of Zn, an extended sampling time of at least ten days to quantitatively assess absorption in humans was proposed. It is assumed that ionic Zn would be absorbed via human hair follicles.

Recommendations

69. For proper analysis of mass balance in dermal absorption studies it is recommended to prolong sampling periods in human and animal studies, especially if the mass balance calculations from pilot studies will reveal less than 90% recovery. It has to be checked which type of dermal samples (excised or reconstructed) would be sufficiently stable to be used in *in vitro* dermal absorption studies with a prolonged post exposure period.

Analysis and Discussion

Testing Methods for dermal Absorption Assessment

Q1: Are OECD TG 427/428 adequate?

70. Based on the observations on ZnO in humans *in vivo*, it was concluded that observation time in OECD TG 427/428 may not be adequate, as increased blood levels were first detected *in vivo* on day 2 using a very sensitive method, and these blood levels increased until the end of the study. The sampling periods of at least 10 days may be required for human skin. However, a sampling time of 10 days is technically not easy for the *in vitro* method with excised skin. The applicability of human 3D reconstructed full thickness skin models would have to be validated.

71. Nevertheless it should be stated clearly that Zn-ions (ratio $^{68}Zn/^{64}Zn$) and not particles were measured (paragraph. 67). There was no increase of the physiological Zn content and the total amounts absorbed as detected in blood and urine were small when compared with the amounts of natural Zn normally present in the human body. The pH of the outer layer of the skin, the stratum corneum, ranges from 5.4 to 5.9, and in such an environment, ZnO particles could partially dissolve. This is supported by the parallel test group with the bulk ZnO, in which also an increase in the ratio $^{68}Zn/^{64}Zn$ in blood was measured.

72. It was agreed, that any study with shorter observation time should be interpreted with great care, until more confidence in the applicability of the TG427 and TG428 for particulates is gained. A corresponding recommendation should be published, based on a study with a partly soluble particle. Currently most animal data do not support the penetration of particles through healthy skin.

73. It is proposed to initiate further expert discussion whether for risk assessment purposes, the amount of nanomaterials penetrated into the upper skin layers in a TG 427/428 study might be considered as potentially absorbable in a worst-case approach. In this context, the turn-over of stratum corneum should be taken in consideration, as this may justify excluding the first five or more tape strippings from the calculation of absorbable dose. In addition, it was noted that the *in vitro* model (TG 428) cannot yet mimic the mechanical processes, such as flexation, that potentially influence translocation of chemicals and particulates. Corresponding method development and validation should be supported.

Q2: Is there a need to distinguish between chemicals and particulates?

74. The guideline should be adapted to accommodate nanomaterials.

Recommendations for further testing and discussion

75. More data is needed to evaluate the appropriateness of the following proposal: A two tiered approach might be considered in which a short-term study is performed first. When there is significant penetration into the skin including the stratum corneum (again: first five tape strippings of stratum

corneum not to be considered!) and hair follicles (e.g. less than 90% removable from skin), an extended follow-up (e.g. ~10 days) should be done to determine whether the amount in skin can become systemically available.

It should be evaluated whether for other materials than ZnO, a "delayed" permeation can also occur. Factors to be taken into account while planning and conducting longer-term dermal translocation studies include the choice of animal species and the availability of sufficiently sensitive detection methods. It was noted that further relevant data has recently become available on silver nanoparticles.

ALTERNATIVE METHODS

Co-chairs: Mario Goetz (Germany) and Karin Wiench (BIAC)

76. Dr. Agnieszka Kinsner-Ovaskainen (EC, JRC) presented a comprehensive review of the advantages and experimental challenges faced when using *in vitro* methods for investigation of cellular uptake, translocation across physiological barriers and cell and tissue responses to manufactured nanomaterials ('*In vitro* barrier models and dosimetry *in vitro*').

77. It was mentioned that with the exception for dermal absorption testing (TG 428), there are no further validated guidelines based on *in vitro* methods to estimate the translocation effects of chemicals including nanomaterials through biological barriers. It was suggested to develop reliable methods to investigate nanomaterial translocation through the lung epithelium, the dermal barrier, endothelial cell layers and basal membranes from blood vessels in placenta, brain, and gonads, as well as through the intestinal barrier. Sufficient 'functional characteristics similarity' between *in vitro* and *in vivo* is crucial here. Currently two exposure systems for cells grown on permeable inserts are mainly used: 1) exposure of submersed cells in culture medium and 2) exposure of cells cultured at the air-liquid interface (e.g. CULTEX®; VITROCELL®). Importantly, to reproducibly quantify nanomaterials translocation *in vitro*, sensitive analytical methods are necessary to detect very small amounts of nanomaterials inside the tissue and in the receptor fluids. The best approach might be to use radioactive tracers attached to or embedded inside the nanomaterials under investigation.

78. A commonly used intestine model comprises human colorectal adenocarcinoma cells (Caco-2) differentiated in culture inserts for 21 days to form highly polarized epithelia of enterocyte-like cells. Caco-2 cells were utilised in pharmaceutical industry for studying nano-formulations of drugs. The differentiated cells bear tight junctions, and well developed microvilli on the apical membrane. Nowadays, several co-cultures of Caco-2 cells with one or more other cell types are being developed to better mimic the *in vivo* intestine. Cultures were established consisting of Caco-2 and mucus secreting goblet HT-29 cells that are able to produce mucus which completely covers the cell monolayer and is 2-10 µm thick. In addition, triple cell cultures including immune cells can be used to mimic inflammatory reactions in the intestine. Co-cultures of Caco-2 cells with lymphoma derived cell lines (Raji cells) induce differentiation of the epithelial cells into M cells. Permeability studies using polystyrene NPs suggest that M-cells are responsible for NPs penetration across the barrier and that the mucus layer affects absorption of nanomaterials.

79. When translocation of ^{56}Co-SiO_2 NP were investigated *in vitro* using differentiated Caco-2 cells, a maximum of 2% mass was determined to cross the intestinal model barrier. The used of radiolabelled nanomaterials allows to calculate a "mass balance".

80. However, it has to be kept in mind that *in vivo* studies normally compare the externally administered dose with the absorbed dose in the blood. Thus, the dose administered *in vivo* is not comparable to the *in vitro* applied dose unless the true *in vivo* dose inside the gut is determined. In addition, the presence of food, bacterial gut microflora and digestion processes (which all are not present *in vitro*) may change the particle physicochemical characteristics (e.g. size and surface, dissolution). In saliva, the stomach and small intestine a lot of the nanomaterial will be modified so that doses reaching the

small intestine may be reduced, hence only a smaller amount would be available to translocate through the intestine to the portal vein.

81. As a result, selected intestinal *in vitro* models can be used to estimate the potential for particle translocation through intestinal cells. The degree of prediction of an *in vivo* effect in a single, well selected *in vitro* test system is however low, since *in vivo* exposure covers multiple digestive steps and organs in the whole digestive tract. Indeed, these multiple digestive steps are on the way to be modelled *in vitro* as well (see contribution by Professor Dr. A. Lampen in session I).

82. As for lung epithelial barrier models, several systems are proposed including primary cells from human tracheal and bronchial epithelium that also produce mucus and even ciliary movement. As well, triple co-culture models constituted by dendritic cells, epithelial alveolar or bronchial cells and macrophages derived from human peripheral monocytes are used to create an immune competent system.

83. For mimicking realistic scenarios, exposure in the air-liquid interface cell exposure systems (ALICE, e.g. CULTEX®; VITROCELL ®) is preferred although usually only short exposure times can be investigated, but the extracellular dose can be better controlled.

84. As for specialized barriers, several *in vitro* models of the blood brain barrier and the blood placenta barrier are under development which integrate various vascular cell types and cells of neural and trophoblast origin, respectively. Most models are in the advanced developmental stage, and results using nanomaterials are avidly expected. The most crucial part in *in vitro* nanotoxicology is the definition of the dose and its corresponding dose metrics.

85. Most nanomaterials are insoluble in culture medium and their transport is diffusion and gravitational sedimentation-driven[11]&[12]. When introduced into the cell culture media NMs diffuse, agglomerate and sediment as a function of different factors, such as media density and viscosity, NM size, shape, surface chemistry, charge, density and nominal concentration. The effective dose of NMs that reach the cellular layer strongly depends on the kinetics of NM deposition and is very often significantly different from the initial, administered dose. During *in vitro* exposure, nanomaterials that come into contact with cells are a mixture of agglomerates of different sizes to an extent very much depending on the nanomaterials properties and the used *in vitro* medium. Therefore, in order to reliably establish the biologically active or toxic dose (i.e. the amount of nanomaterials that effectively reach the cells), a proper physicochemical characterisation of NMs in the culture condition and the NMs transport in the cell culture medium must be taken into account.

86. The influences of the size, aggregation/agglomeration state and protein corona on the mass fraction deposited on cells *in vitro* were systematically investigated at a constant concentration of 20 µg Au NP per ml cell culture medium. 80 nm Au nanoparticles deposited in much higher number (% of administered amount) in the presence of cells, as compared to 20 nm Au nanoparticles which were deposited *in vitro* in very small amounts and thus only accidentally came into contact with cells within 72 h exposure time. Currently, mathematical models calculating sedimentation behaviour of nanomaterials are

[11] E. C. Cho, et al. The effect of sedimentation and diffusion on cellular uptake of gold nanoparticles. Nature Nanotech. Vol: 6, Pages: 385–391 (2011)

[12] Limbach LK, et al.: Oxide nanoparticle uptake in human lung fibroblasts: effects of particle size, agglomeration, and diffusion at low concentrations. Environ. Sci. Technol. 2005, 39:9370-9376.

developed (e.g. ISDD model[13]). With a properly addressed dosimetry, the *in vitro* models may become relevant in weight of evidence approaches in risk assessment.

87. In summary, for the skin, colon, the bronchial and alveolar cell types suitable models for studying translocation of soluble chemicals already exist. Nowadays, the cell models can even mimic more realistic exposure scenarios by including mucus producing cells and immune cells. The *in vitro* models might be applied for the analysis of nanomaterials translocation, as long as care is taken of the *in vitro* biokinetics. The latter is defined as the whole of physicochemical changes, the diffusion, sedimentation and finally possible uptake by the cells during the course of the test. The internal dose is particle size and time dependent and can be affected by components of the test system such as serum proteins.

Analysis and discussion

Q1. How could risk assessment, in principle, be informed by in vitro methods for biokinetics? Which endpoints may be served?

88. There was agreement that, currently, the available methods are not appropriate for assessment of nanomaterial biokinetics and available data should be used with caution only. This includes any first tier considerations (worst-case approaches).

89. Models for GI tract and lung are most advanced. Blood-brain-barrier models are not as highly developed and are not human based. For the placenta, mainly the ex-vivo perfused placenta models are available, and the *in vitro* models are not considered appropriate.

90. However there is a will and a need to further develop these models. A list of priority issues to be considered (e.g. dosimetry) and addressed in the future programme should be generated.

Q2. Which in vitro barrier models can currently inform risk assessment and how?

91. Currently, many of these models are used to improve our understanding about mechanistic aspects involved in translocation of nanomaterials across biological barriers.

Q3. Which further developments are considered necessary? Are there any priorities?

92. There may not be one single method to cover all nanomaterials, but specific assays for specific types of nanomaterials could be relevant. *In vitro* barriers seem to be leakier than their *in vivo* counterparts. Also the dose per unit area seems to be far higher than realistic *in vivo* exposure doses. But recent developments are very promising and should be followed up (strong support).

93. The methods should also be applicable to non-radiolabelled particles (e.g. particle bombardment not usually available in industry). Readily available techniques that do not interfere with particle properties are preferred. Extrinsic labelling should be avoided.

94. For models of the lung, air-liquid interface models were generally given preference as with those devices actual dose is more easily controlled and relevant aerosols can be applied.

[13] Teeguarden JG et al.: Particokinetics in vitro: dosimetry considerations for in vitro nanoparticle toxicity assessments. Toxicological Sciences 2007, 95:300-312.

Q4. (Session 4b). Do we need guidelines/guidance for in vitro biokinetic testing and use of the data obtained?

95. Development of general guidance should be considered once clarity is reached with regard to open issues in dosimetry.

96. In the meantime, the initiation of further round robin testing programmes with existing and newly developed/refined models were suggested, which should provide an improved basis for guideline development.

97. As a crucial consequence, nanomaterial exposure scenarios *in vitro* would have to be monitored by measuring the nanomaterials quantity and size distribution on and inside the cells.

98. For kinetic sedimentation and translocation studies, sensitive analytical methods are needed to quantify the nanomaterials in the tissue model and in the acceptor fluids. However, for the future other modes of exposure (e.g. microfluidic or in rotation) might be more useful than relying on sedimentation and diffusion only. The modelling of the placenta and brain barriers is still in an early developmental stage. Since placenta is also changing during pregnancy, it is not yet clear how to mimic the placental changes *in vitro*. Thus, it is too early to define guidance for the use of *in vitro* model outcomes for risk assessment purposes; however some models may soon become suitable for assessing translocation potentials of nanomaterials through epithelial barriers. The development of harmonised protocols and subsequent round robin testing is encouraged.

TESTING METHODS FOR TOXICOKINETICS FOLLOWING INHALATION

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99. This session included, three presentations were made from the viewpoints of different *in vivo* testing methods such as intratracheal administration, short-term inhalation and 28 days inhalation for evaluating toxicokinetics after inhalation exposure to nanomaterials. Then, the usefulness of the methods and the approach to the future development of guidelines/guidance on toxicokinetics were discussed.

100. In the first presentation, Dr. Masashi Gamo (Japan) showed results of toxicokinetics studies using intratracheal administration conducted in a Japanese research project "Development of Innovative Methodology for Safety Assessment of Industrial Nanomaterials" during 2011-2015.

101. In the comparison between intratracheal administration (IT) and inhalation exposure (IH) studies on nickel oxide (NiO) and C_{60}, it was shown that half-lives of clearance of the nanomaterials from the lung were comparable when the deposited amounts in the lung were similar between IT and IH.

102. Another comparison between different TiO_2 nanomaterials using IT showed that a TiO_2 nanomaterial with surface coating of $Al(OH)_3$ depressed the clearance rates from the lung. As his conclusion, these toxicokinetics studies suggested that IT studies can produce initial characterization of toxicokinetics of nanomaterials.

103. He also pointed out that standardization of procedures, appropriate sample preparation and dose settings, and adequate post-administration observation period, e.g. 3 months were essential for using IT for initial characterisation of hazard and toxicokinetics of nanomaterials.

104. It has been suggested by Dr. Gamo during his presentation that the IT study can produce initial characterization of toxicokinetics of NMs, which comparable to IH study. It can also enable us to compare the toxicokinetic characteristics between NMs.

105. It has been mentioned that there is a lack of standard procedures for IT which made it difficult to compare the test results produced by different facilities.

106. For use of IT for initial characterization of hazard and toxicokinetics of NMs, it is essential to standardize the procedures, to have the adequate sample preparation and dose setting as well as an adequate observation period after administration (e.g. 3 mo.).

107. In the second presentation, Dr. Karin Wiench (BASF) introduced toxicokinetics study using short-term inhalation study.

108. The Short-term Inhalation Study of Nanomaterials (NSTIS) was developed within the European *NanoSafe2* and the German *nanoCare* project to readily assess the effects and burden of nanomaterials in

the body[14]. NSTIS is a 28-day study with five days of inhalation exposure and three weeks post-exposure observation. Exposure and biological read-outs were optimized for nanomaterials and - unlike the standard 28-day inhalation studies[15] – the protocol includes the measurement of lung burden and extra pulmonary tissue levels and the clearance within the three week window.

109. Meanwhile more than twenty nanomaterials have been tested with NSTIS[16] and a recent OECD paper has analyzed the likeness of the lunge effects observed in NSTIS with effects observed in subchronic and chronic inhalation studies[17].

110. The lung deposition of different nanomaterials in the lung typically ranged between five and eighteen percent and the clearance were between zero and more than fifty percent within three weeks. High deposition and low clearance rates usually raise concerns and trigger prioritization and further tests of the respective nanomaterial.

111. The nanomaterials were generally located in the lung and draining lymph node, but no translocation to other tissues was found for most nanomaterials. A polymer-coated silica particle was, however, detected in the spleen and product development was terminated (further targeted toxicity testing would have been the alternative).

112. Lung deposition and persistence as well as translocation of inhaled nanomaterials vary for different materials and their assessment is indispensable for understanding and assessing their toxic effects. The NSTIS demonstrates how these – however limited – toxicokinetic data can be included in an inhalation study to provide data on the effects as well as organ burdens in an efficient way.

113. In the third presentation, Dr. Jos Bessems (EC) introduced an amendment to the 28-day inhalation toxicity study with biokinetics.

114. Recommendations were made for adapting current guidelines and the guidance document for inhalation toxicity testing of nanomaterials that include deletion of a lower cut-off of the size range and minimal mass concentrations. Specifically, the expert group mentioned the need for careful aerosol generation and characterization. Most experts agreed more explicitly to incorporate the application of biokinetics into the guideline and detailed pathology of the brain and other parts of the central nerve system, while bronchoalveolar lavage fluid analysis should be included as a mandatory requirement.

115. The test atmosphere generated should have workplace characteristics, as much as possible.

116. Dissolution behaviour of the test substance should be assessed in physiological fluids mimicking various lung-specific pH environments (neutral, acid).

[14] Ma-Hock L, Burkhardt S, Strauss V, Gamer A, Wiench K, van Ravenzwaay B, Landsiedel R (2008): Development of a short-term inhalation test in rats using nano-titanium dioxide as a model substance, Inhalation Toxicology, 21:102-118, 2009.

[15] OECD test guidelines no. 412 (http://www.oecd-ilibrary.org/environment/test-no-412-subacute-inhalation-toxicity-28 days study_9789264070783-en)

[16] Landsiedel R, Ma-Hock, L, Kroll, A, Hahn D, Schnekenburger J, Wiench K, Wohlleben W (2010): Testing metal-oxide nanomaterials for human safety. Advanced Materials, Vol. 22: 2601-27

[17] Klein CL, Wiench K, Wiemann M, Ma-Hock L, van Ravenzwaay B, Landsiedel R.: Hazard identification of inhaled nanomaterials: making use of short-term inhalation studies. Arch Toxicol. 2012 Apr 25. [Epub ahead of print] PMID: 22532024

117. Data analysis should include interpretation of aerosol characteristics, NOAEL, risk assessment implications, mode of action and a strategy for dosimetric extrapolation to humans.

118. Retention and clearance should also be considered for inclusion in repeated dose studies.

119. Both the study design (dose selection) and the selection of the post exposure recovery period should take into account the kinetic behaviour at non-overload and overload conditions. Animal numbers and study costs can be significantly reduced by executing hypothesis-based (PBPK modelling) repeated exposure inhalation studies.

120. Additions to the inhalation test guidelines such as TGs 412 (e.g. as 'Optional testing on biokinetics' as a separate paragraph after paragraph 44) and 413 could occur in the area of nanomaterial biokinetics. While detection methods for some nanomaterials are not readily available and there are few studies currently in this area, there is a need to understand the absorption and organ distribution kinetics (down to the level of cellular uptake) and retention of nanomaterials in the lung and other organs but also the extent and impact of agglomeration/de-agglomeration as well as solubility issues and secondary formation of inhaled particles. Additions to OECD Guidance that request minimal kinetic and retention parameters on an optional basis should be considered. These should be accompanied by the definition of quality standards for detection methods in tissues and acceptable detection limits (based on the progress in the analytical field).

121. Benefits of including minimal biokinetic parameter sampling in OECD TGs 412 and 413:

- Determination of lung deposition and clearance of inhaled substances.
- Improve the risk assessment quality. With the availability of 'internal/systemic dosimetry anchoring points', uncertainty with respect to various common risk assessment extrapolations could be reduced.
- Generation of sufficient information in TG 412/413 to conclude whether, upon inhalation, particles or fibres (including released molecules/atoms/ions) become systemically available or not. This relates to the issue of a post-administration observation period. Depending on the regulatory framework, proof of non-relevant absorption/bioavailability[18] could result in waiving of a longer-term study such as the 90 days inhalation study in TG 413.
- Where inhaled substances (particles/fibres or their 'products') are systemically available, indications on the distribution and the rate of whole body clearance via faeces or urine should be determined. This would provide screening level information as to whether whole body clearance is relatively rapid or whether there is an apparent accumulation potential.

122. Suggested base set on biokinetic parameters for inclusion in OECD TGs 412 and 413:

- Matrices to sample (blood and/or tissue): Needs are very much dependent on the expected/known ADME[19]/toxicokinetic characteristics of the nanomaterial. Liver, spleen, brain (including the olfactory bulb and the hippocampus), kidney, bone marrow and lung are typical tissues to which nanomaterials distribute. Lung is strongly advised, e.g. in order to set external exposure – lung dose – response continuum and enable a check for 'internal dose'-linearity.

[18] Criteria to assess 'non-relevant' or 'negligible' absorption and/or bioavailability should be established in the specific regulatory framework.

[19] Absorption, Distribution, Metabolism, and Excretion.

Especially in the area of nanoparticles, it may be interesting to see whether relative absorption may be decreased at high exposure concentrations as a result of airborne formation of aggregates. Urine and faeces sampling could also be considered (total element analysis) in order to provide preliminary data with respect to clearance rates and clearance routes.

- Time points to sample: Poorly- and non-soluble particles/fibres often have slow clearance rates; as initial sampling points, 7 days and 28 days after exposure may be considered. Later sampling points may be in the range of 28 – 90 days post-exposure, depending on the post-administration period chosen.

- Microscopic or electron-microscopic observation of the nanomaterials in tissues. With this observation, qualitative information can be obtained even when quantitative determination of the nanomaterials in the tissues is difficult, and also suggestions for mechanisms of adverse effects of the exposure may be given.

Animal welfare considerations

123. Inclusion of basic biokinetic parameter sampling in a study on rodents may result in more stress for the animals or even in some more animals (satellite group). However, there are advantages with respect to the 3Rs (Replacement, Reduction and Refinement of animal testing) as well. By inclusion of basis biokinetic parameters in the study design of the TG 412/413, the additional information could improve human risk assessment and may decrease the need for additional and/or new studies. Another advantage is a preliminary check for dose-linearity. Especially in the area of nanoparticles, it may be interesting to see whether relative absorption may be decreased at high exposure concentrations as a result of airborne formation of aggregates. Lastly, as the quality of the toxicity (health effect) data will increase almost certainly, the chance of a need for follow-up toxicity study/studies to fill in remaining data gaps may very well decrease.

CONCLUSIONS

124. The intratracheal administration and the short-term inhalation test (NSTIS) may be useful for examination of acute exposure and for comparative studies. They should be further developed and experience gathered but there is currently no need to initiate a TG process.

125. The current process of the amendment of TG 412 (28-day inhalation toxicity study) by inclusion of toxicokinetic parameters is welcomed and supported by the expert meeting.

126. The toxicokinetics guideline TG 417 is not suitable for nanomaterials. With regard to the oral exposure path it is recommended to revise it in order to make it appropriate for nanomaterials. With respect to inhalation exposure a new guideline is recommended or the addition of a part 2 to TG 417 for inhalation exposure may be the better alternative.

127. TG 427/28 in combination with GD 28 and 156 are in general applicable for nanomaterials. When updated, some amendments, for example, regarding longer observation periods with nanomaterials should be mentioned.

128. Participants are encouraged to take the lead by preparing SPSFs as initial step for preparing test guidelines and guidance documents.

129. Physiologically Based Pharmacokinetic (PBPK) modelling approaches may serve as very useful tools to interpret and predict hypothesis testing of toxicokinetic behaviour of nanomaterials and should be further developed.

130. Progress on *in vitro* methods in particular with respect to barrier models is encouraging.