Session Nanotoxicology[1]

Linz 2010, 16th Congress on Alternatives to Animal Testing, 3rd September 2010

In the fully occupied reception hall of the Johannes Kepler University, Linz, Austria, **Sabine Umbricht-Vonlanthen**, Director of the Foundation Animalfree Research, welcomed the audience and gave an outline of the Foundation's motivation for organising a Special Session on Nanotoxicology (co-chaired by **Ursula G. Sauer**, Scientific Consultancy – Animal Welfare, D-Neubiberg and **Robert Landsiedel**, BASF, D-Ludwigshafen): the increasing importance that nanotechnology is being given by research and economic policies, the animal welfare concerns related to the new technology and also the upcoming European Commission's new EU Strategic Nanotechnology Action Plan 2010-2015.

Robert Landsiedel, Head of the Group of Experimental Toxicology at BASF SE, D-Ludwigshafen, held the session's state-of-the-art lecture on *The use of alternative methods for toxicity testing of nanomaterials*. He presented an overview on the mechanisms of uptake and effects of nanomaterials (NM) in the body. Primary effects of NM can be inflammation, formation of reactive compounds, ion release or direct interactions of NM with cellular structures. These primary effects might lead to secondary toxic effects. However, no new nano-specific toxic effect has been observed. Therefore, in principle, established testing methods are considered adequate to investigate NM effects, but they might require adaptations, e.g. regarding test substance application (Schulze et al., 2008), and might also need to address new concerns arising with the development of the new field of nanotechnology. For instance, *in vitro* dermal absorption of sunscreen nanoparticles was first evaluated using normal porcine skin and it was found that nanoparticles do not penetrate this. As a result it was requested to test whether they would penetrate sunburned skin – which they also did not. Experience gained with *in vitro* genotoxicity studies (Landsiedel et al., 2009) revealed the necessity to reproducibly disperse NM in the culture medium and to characterise particle size distribution in test samples to ensure meaningful test results. An *in vitro* testing strategy for inhalational toxicity testing, an important endpoint in the hazard assessment of NM, is not yet available. With the aim to at least reduce and refine the conventional 28-day study, and possibly also the 90-day study in rats, a 5-day inhalational study was compiled. This test includes organ histopathology as well as cytological and biochemical investigations of the broncho-alveolar lavage fluid at the end of the study phase. During the development of this 5-day protocol, relevant and predictive biological endpoints were selected among more than 80 parameters (Ma-Hock et al., 2007; Ma-Hock et al., 2009). Results from 5-day inhalational studies with different NM showed inflammation of the lung as the main effect of biopersistent particles; the potency of different particles, however, varied over two orders of magnitude. In consequence, the specific mechanisms of the pulmonary effects of NM could be focused on and further tested *in vitro* (Landsiedel et al., 2010). Robert Landsiedel highlighted an integrated, stepwise testing strategy for NM human health effect assessment developed jointly by the Netherlands Environmental Assessment Agency (RIVM), the European Commission's Joint Research Centre (JRC) and BASF within the NAPIRA network. This integrated testing strategy is currently under discussion at the OECD Working Party for Manufactured Nanomaterials. In its first tier, *in vitro* local effects and primary biological effects on the one hand and the kinetic translocation of NM on the other hand are tested separately. Due to this splitting, *in vivo* testing can be avoided in the initial stage of the testing strategy. If NM do not show any biological effects *in vitro*, they are unlikely to have effects *in vivo*; and further toxicity testing is considered unnecessary (but there might be a need for bioaccumulation testing). Likewise, if tier 1 kinetic evaluations do not reveal NM translocation, they are assumed to be unlikely to have systemic effects, and again further studies for systemic effects are not necessary. While *in vitro* methods for the tier 1 testing are under development and validation, the *in vivo* 5-day inhalation test could serve as an interim study for two purposes: to make the tiered testing strategy accessible today (instead of having to wait for validation of the complete

[1] funded by Animalfree Research, Zurich, Switzerland

battery of *in vitro* methods) and to generate reference data for the development and validation of *in vitro* assays. Robert Landsiedel concluded his presentation by referring to the grouping of substances as another potential means to further reduce the need for animal testing. While it has not yet been possible to group NM according to their chemical structure or their physical properties, he suggested striving to group NM by their biological activity, which again can be performed using *in vitro* test methods.

Mario Götz, Head of the Molecular Toxicology Unit at the German Federal Institute for Risk Assessment, BfR, D-Berlin, and Co-Chair of the Steering Group 7 (SG7) of the OECD Working Party on Manufactured Nanomaterials (WPMN), elucidated *The role of alternative test methods in nanotoxicology from the BfR perspective*. He presented an overview of the activities of the SG7, which has been entrusted with the task to determine which existing or new *in vitro* test methods could be suitable for hazard assessment of NM. Existing, non-animal OECD Test Guidelines (TG)[2] cover the endpoints skin absorption (TG428), skin corrosivity (TG430, TG431, TG435), phototoxicity (TG432), genotoxicity (TG471, TG473, TG476) as well as the recently adopted Test Guidelines for eye and skin irritation testing (TG 437, TG438 and TG439, respectively). A further *in vitro* genotoxicity test (TG487) has also recently been adopted. Mario Götz discussed the state-of-the-art and knowledge gaps regarding the applicability of these accepted Test Guidelines for NM testing. Promising, new *in vitro* approaches to test NM, also under the OECD Sponsorship Programme for the Testing of Manufactured Nanomaterials[3], address endpoints such as cell viability, penetration through biological barriers, target organ toxicity – including neurotoxicity, cardiotoxicity, immunotoxicity – and the potential of NM to produce inflammation and oxidative stress. Having not yet undergone any formal validation procedure, all these new *in vitro* approaches are currently considered explorative screening assays. To promote their regulatory acceptance, the most relevant *in vitro* methods covering those cell types that are most sensitive to NM should be prioritised and recommended for further development and validation. In this context, NM sample preparation and dosimetrics should be standardised. An important endpoint currently lacking *in vitro* alternatives is repeated dose toxicity. Mario Götz also emphasised the need to establish an integrated testing strategy for meaningful NM toxicity data collection.

After these overviews on non-animal NM testing strategies, the following two presentations explained two specific co-culture models for determining barrier effects of NM at the pulmonary and intestinal barriers, respectively.

Christina Brandenberger, formerly Working Group of Peter Gehr and Barbara Rothen-Rutishauser at the Institute of Anatomy, University of CH-Bern, currently Lung Biology Laboratory at the Columbia University, USA-New York), presented the *Air-liquid exposure of gold nanoparticles to study effects, uptake and intracellular distribution in a human 3D epithelial airway model*. 15 nm gold nanoparticles, which show promising characteristics for application in biomedicine, were analysed in a human epithelial airway triple cell co-culture model (Rothen-Rutishauser et al., 2005), exposed in a newly designed air-liquid interface cell exposure (ALICE) system (Lenz et al., 2009). The co-culture model consists of human alveolar epithelial A549 cells combined with human blood-monocyte derived macrophages on top and dendritic cells underneath, thereby simulating the most important barrier functions of the lung epithelial airway. The cells receive culture medium from below and are exposed to the NM at the air liquid interface on top. For exposure, the cell culture plates are placed into the isolation box of the exposure system. An aerosoliser generates a homogeneous aerosol from the particle suspension and gravitational settling allows an even particle deposition at the air-liquid interface of the cells. Thereby a dose-controlled deposition of gold NM aerosols on top of the cells can be administered, which mimics *in vivo* aerosol inhalation very realistically. The dose deposited on the cells can be controlled with a quartz crystal microbalance, providing proof-of-principle evidence of the reliable and homogenous deposition of non-agglomerated particles. In comparison, when NM were deposited onto cells in suspension media, a large proportion of the NM agglomerated and particle uptake was approximately 7 times lower due to diffusional particle loss in suspension (Brandenberger et al., 2010a). After exposure in the ALICE system, the gold nanoparticles were detected in all cell types of the triple co-culture, indicating their translocation through the epithelial barrier also to lower cell layers. Cellular effects of the gold nanoparticles were determined, such as protein and gene expression and oxidative stress (Brandenberger et al., 2010b), as well as the different endocytic mechanisms of uptake of NM into the cells (Brandenberger et al., 2010a). A pre-stimulation with lipopolysaccharide (LPS) served to further investigate the effects of particles under inflammatory conditions. In the studies, the gold nanoparticles did not cause adverse effects on the cells.

Eva-Maria Collnot, Group Leader at the Helmholtz Institute for Pharmaceutical Research Saarland, D-Saarbrücken, Head Claus-Michael Lehr, presented an *In vitro model of inflammatory bowel disease for drug formulation testing and screening*. The motivation for developing this *in vitro* model was driven by both ethical and scientific problems related to the corresponding animal models. The co-culture model consists of Caco-2 intestinal epithelial cells in combination with human blood-derived macrophages and dendritic cells, which are embedded in a collagen matrix (Leonhard et al., in press). The addition of the pro-inflammatory cytokine interleukin-1β triggers inflammatory reactions. Measurement of pro-inflammatory markers, of TEER-related changes in the

[2] http://lysander.sourceoecd.org/vl=10310848/cl=29/nw=1/rpsv/cw/vhosts/oecdjournals/1607310x/v1n4/contp1-1.htm (all websites were accessed in September 2010)

[3] http://www.oecd.org/document/47/0,3343,en_2649_37015404_41197295_1_1_1_1,00.html

barrier function and also of changes in mucous production revealed that the model reflects pathophysiological changes as well as recovery from these. When NM are deposited on the cells, a higher uptake by inflamed cells in comparison to non-inflamed cells can be observed. While the *in vitro* intestinal model was initially developed to determine the *in vitro* effects of pharmaceutical substances, the research group has recently joined an EU FP7 Integrated Project, InLiveTox[4], in which the applicability of the model to test the unwanted translocation of engineered NM through the intestinal barrier is being investigated.

After these presentations focusing on human hazard assessment, the final presentation addressed ecotoxicological aspects of NM.

Anne Kahru, Head of the Laboratory of Genetics at the National Institute of Chemical Physics and Biophysics, Tallinn, Estonia and President of the Estonian Society of Toxicology, presented an approach to the *High-to-medium-throughput evaluation of the ecotoxicity and toxicological profiling of synthetic nanoparticles*. She highlighted that, in addition to natural and anthropogenic NM, there are synthetic NM that may end up as environmental contaminants, and that there are vast data gaps as to the adverse effects of NM, especially in the area of ecotoxicology (Kahru and Savolainen, 2010). To address these data gaps, Anne Kahru's research group has begun evaluating the hazard of nanoparticulate ZnO, CuO, TiO_2 and Ag as well as C60-fullerenes in standard ecotoxicity organisms, such as algae, daphnids and bacteria, but also in less frequently used model organisms, such as yeast and protozoa. All tested NM were revealed to be at least harmful, if not toxic or very toxic, to the selected organisms (Kahru and Dubourguier, 2010). In consequence, Anne Kahru recommended applying the precautionary principle as long as proper ecotoxicity hazard evaluations have not yet been performed. Further studies explored the mechanisms of NM ecotoxicity. With few exceptions, solubility seems to be the key determinant of the toxicity of metal-containing NM. Thus metal NM can exert toxic effects by releasing metal ions, which enter target cells and target organisms. Finally, Anne Kahru described the tailored construction of different strains of recombinant luminescent bacteria and their use for the rapid mechanistic profiling of metal solubilisation and reactive oxygen species related ecotoxicity of NM. These studies are being continued in the FP7 funded EU Project "NANOVALID" (Development of Reference Methods for Hazard Identification, Risk Assessment and LCA of Engineered Nanomaterials).

In the second part of the Nanotoxicology session, all presenters of lectures participated at the *Round Table* "*Towards non-animal testing strategies in nanotoxicology – what needs to be done and who will do it?*" moderated by Ursula G. Sauer. They were joined by **Cyrille Krul**, the Coordinator for Research Programmes on 3Rs at the Netherlands Organisation for Applied Scientific Research (TNO) in NL-Zeist and Professor for Alternative Methods at the University of Applied Sciences, NL-Utrecht (presenting a poster at the Linz Congress, entitled *Alternative thinking on hazard assessment of new materials: nanomaterials and 3Rs*).

In her introductory statement, Cyrille Krul acknowledged the importance of integrated testing strategies for a meaningful NM hazard assessment. She expressed hope that such intelligent testing strategies for different types of NM could be agreed upon and that NM grouping would be possible, which would obviate testing every single NM. Cyrille Krul stressed that closing the knowledge gaps on the mechanisms of NM effects requires a combined effort from industry, academia and regulators.

Throughout the discussions there was unanimous agreement that the RIVM/JRC/BASF integrated testing strategy provided a meaningful and valuable approach to NM hazard assessment. Applying such a tiered testing strategy would enable screening out NM posing no concern with *in vitro* assays, an approach already commonly accepted for mutagenicity testing of "bulk" chemicals. This would considerably reduce animal testing and meet economic interests.

Robert Landsiedel mentioned the development of *in vitro* models for pulmonary toxicity testing, further adaptations of the *in vitro* genotoxic models and further developments of *in vitro* models on the kinetic translocation of NM as possible next research goals to further reduce the need for *in vivo* test methods in the tiered testing strategies. Asked about the industry's commitment to ecotoxicity assessments, Robert Landsiedel referred to BASF's studies on aquatic toxicity (Wiench et al., 2009) and explained that his company's prioritisation for substance evaluation is made according to the respective possible exposure. Accordingly, workers are the first and most likely to come into contact with new substances. Exposure of the environment would only occur if particles were actually released from the composite materials or surfaces. This has recently been studied at BASF (publication in preparation). Anne Kahru re-emphasised that knowledge on ecotoxicological testing of NM is still at a very early stage.

The issue of NM in food – the sector with the highest number of patents related to NM – was addressed briefly. Solubility was recognised as a factor that should be considered when determining the effect of NM in food, since soluble NM would behave very much like a solution of the same substance, although the bioavailability could be different.

The airway epithelium and intestinal models presented by Christina Brandenberger and Eva-Maria Collnot were lauded as promising methods to test barrier effects of NM. Even though they were originally developed to study NM for pharmaceutical applications, it was recommended to develop them further for nanotoxicological testing. Regarding necessary steps for standardising the ALICE system, Christina Brandenberger pointed

[4] http://www.inlivetox.eu

out that such a time-consuming effort would require collaboration with industry. It would also be important to ensure a reliable and standardised source of monocytes for the cell culture, since this currently relies upon individual blood donations. Nevertheless, she confirmed that the ALICE system is easy to handle and can be adapted for different applications. Therefore it should be a useful tool for NM hazard assessment.

Asked about the OECD WPMN's procedure for selecting new *in vitro* approaches for testing strategies and test guidelines, Mario Götz described that the SG7 relies on receiving scientific information and on data from the OECD Sponsorship Programme. He underlined the importance of scientists forwarding relevant information, also on methods entering pre-validation and validation studies, to their respective National Coordinators of the OECD WPMN and to the Co-Chairs of SG7, Christoph Klein (JRC) and himself (BfR).

Regarding the validation of test methods, there was agreement that the lack of relevant data against which to validate new methods poses a serious problem. Taking the example of the 5-day inhalational study, Robert Landsiedel pointed out that, so far, altogether only a handful of long-term inhalation studies for NM have been published. However, so far all comparisons confirm that the main effect of inhaled biopersistent NM is an inflammatory reaction in the lung. Therefore the test system is considered highly predictive and parameters examined in the 5-day inhalation studies offer an excellent opportunity to develop *in vitro* assays based on the same biological effects. Cyrille Krul added that data gained from 5-day inhalational studies, not only with the conventional parameters but also with e.g. cytokines and toxicogenomic analysis, correlate well with effects observed in 28-day studies. This might also provide a good approach for studying longer-term effects, even though further validation of this method is required.

Mario Götz explained that the current OECD testing programme is explorative in nature. So far, the OECD does not see the need for validating existing *in vivo* or *in vitro* OECD Test Guidelines to confirm their applicability for NM testing unless the current testing programme reveals major applicability concerns. Regarding the *in vitro* testing of NM as cosmetic ingredients, however, the European Commission's Scientific Committee on Consumer Products requested in 2007 that all *in vitro* tests undergo specific validation studies[5]. Two problems were addressed as currently standing in the way of meeting this request: Firstly, again, there are few data against which to validate the test methods. Furthermore, there are no globally accepted positive controls for NM genotoxicity. Thus it is impossible to determine the value of negative test results. Robert Landsiedel pointed out that milled-down quartz is currently being suggested as a possible positive control, but that this proposal requires further evaluation.

In initiating the final round of the discussions, Ursula G. Sauer reported that the Foundation Animalfree Research had repeatedly tried to invite a representative from the European Commission to the session. Unfortunately, however, this attempt remained unsuccessful. Nevertheless, she saw the Round Table as an opportunity to spell out a message for the European Commission, also in light of the up-coming European Commission's new EU Strategic Nanotechnology Action Plan 2010 – 2015. Accordingly, she asked all participants to name an issue that the European Commission might be invited to address with high priority in order to further promote non-animal test methods for NM hazard assessment.

Christina Brandenberger underlined the importance of ensuring the comparability of *in vitro* studies and, as a result, *in vitro* data. This challenge could be met by establishing guidelines that enable defining and comparing particle types, cell culture systems and exposure systems. Eva-Maria Collnot confirmed this statement. She underlined the importance of characterising NM, both in the test sample and also in their physiological environment as they reach respective target organs or target cells.

Anne Kahru advised to include physicists in the hazard assessment of NM and pointed out that to follow the 3Rs approach, initial stages of NM toxicological profiling could also be performed with invertebrate organisms conventionally used in ecotoxicological testing. Furthermore, she expressed a general recommendation to the European Commission, i.e. to encourage smaller EU projects comprising at the most seven partners, which would allow more effective scientific communication between the partners.

Mario Götz referred to an activity of the current Belgian Presidency of the Council of the European Union, which addresses a regulatory framework for the traceability of nanomaterials[6]. While a product registry for cosmetics containing NM is to be established within the EU in the coming years, this challenge can only be met if NM can indeed be measured in materials and products. This requires the development of robust techniques to identify NM in products. Mario Götz mentioned the hazard identification of NM and the acceptance of an integrated testing strategy as further important challenges.

Robert Landsiedel advised focussing on relevant particles when testing NM. He also emphasised the need for the further development and standardisation of *in vitro* test systems. In the meantime, however, there is no need to apply traditional full-blown standard testing for chemicals. Instead, an integrated testing strategy that streamlines the identification of relevant NM effects and thus reduces animal testing should be used. Moreover, grouping of NM should be pursued as the most promising way to reduce animal testing.

Cyrille Krul recommended taking the opportunity to develop alternative approaches involving the fewest animal test methods possible. Since NM hazard assessment does not have a history of traditional animal testing, this scientific area provides a good opportunity to aim for new options and to change

[5] http://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_099.pdf

[6] http://www.eutrio.be/towards-regulatory-framework-traceability-nanomaterials

paradigms by choosing models that are truly relevant for the human situation.

Stefanie Schindler, Research Assistant to Animalfree Research, closed the session by thanking the participants and by summing up the most important issues, especially mentioning the promising *in vitro* approaches that are already available and the importance of integrated testing strategies and grouping of NM to obviate the need to test every NM in every size, every shape and every suspension. Overall, a coordinated joint effort is needed between industry, academia and regulators to achieve an NM testing strategy that no longer relies on animal testing.

References

Brandenberger, C., Mühlfeld, C., Ali, Z. et al. (2010a). Quantitative evaluation of cellular uptake and trafficking of plain and polyethylene glycol-coated gold nanoparticles. *Small 6(15),* 1669-1678.

Brandenberger, C., Rothen-Rutishauser, B., Mühlfeld, C. et al. (2010b). Effects and uptake of gold nanoparticles deposited at the air-liquid interface of a human epithelial airway model. *Toxicol. Appl. Pharmacol. 242(1),* 56-65.

Kahru, A. and Dubourguier, H.-C. (2010). From ecotoxicology to nanoecotoxicology. *Toxicol. 269(2-3),* 105-119.

Kahru, A. and Savolainen, K. (2010). Potential hazard of nanoparticles: From properties to biological and environmental effects. *Toxicol. 269(2-3),* 89-91.

Landsiedel, R., Schulz, M., Kapp, M. D. and Oesch, F. (2009). Genotoxicity investigations on nanomaterials: methods, preparation and characterization of test material, potential artifacts and limitations – many questions, some answers. *Mutation Res. 681(2-3),* 241-258.

Landsiedel, R., Ma-Hock, L., Kroll, A. et al. (2010). Testing metal-oxide nanomaterials for human safety. *Advanced Materials 22(24),* 2601-2627.

Lenz, A. G., Karg, E., Lentner, B. et al. (2009). A dose-controlled system for air-liquid interface cell exposure and application to zinc oxide nanoparticles. *Particle Fibre Toxicol. 6*, 32.

Leonard, F., Collnot, E. M. and Lehr, C. M. (2010). A 3-dimensional co-culture of enterocytes, monocytes and dendritic cells to model the inflamed intestinal mucosa in vitro. *Mol. Pharmacol.*, in press.

Ma-Hock, L., Gamer, A., Landsiedel, R. et al. (2007). Generation and characterization of test atmospheres with nanomaterials. *Inhalation Toxicol. 19*, 833-848.

Ma-Hock, L., Burkhardt, S., Strauss, V. et al. (2009). Development of a short-term inhalation test in rats using nano-titanium dioxide as a model substance. *Inhalation Toxicol. 21*, 102-118.

Rothen-Ruthishauser, B., Kiama, S.G. and Gehr, P. (2005). A three-dimensional cellular model of the human respiratory tract to study the interaction with particles. *Am. J. Respir. Cell Mol. Biol. 32(4),* 281-289.

Schulze, C., Kroll, A., Lehr, C. M. et al. (2008). Not ready to use - overcoming pitfalls when dispersing nanoparticles in physiological media. *Nanotoxicol. 2*, 51-61.

Wiench, K., Wohlleben, W., Hisgen, V. et al. (2009). Acute and chronic effects of nano- and non-nano-scale TiO_2 and ZnO particles on mobility and reproduction of the freshwater invertebrate Daphnia magna. *Chemosphere 76(10),* 1356-1365.

Ursula G. Sauer