NanoMILE
Engineered nanomaterial mechanisms of interactions with living systems and the environment: a universal framework for safe nanotechnology
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Highlights of key outputs/publications with a focus on the interest for industry & services in the field of nanomaterials

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For up-to-date progress, please go to the "outputs" page on NanoMILE's website (http://www.nanomile.eu*).*

Towards a strategy for grouping and classification of Nanomaterials

A novel approach is proposed to identify interlinked physicochemical descriptors, and on this basis identify overarching descriptors (axes or principle components) which can be used to correlate with toxicity. An example of the approach is provided, using three principle components which we suggest can fully describe each NM, these being the *composition,* the *intrinsic* (inherent) properties of the NM, and *extrinsic* properties (interaction with media, molecular coronas etc.).

Within NanoMILE, we developed a hypothesis that NM toxicity can be predicted as the sum of three "quantifiable" parameters (principle components, PCs) that capture the diversity of modes of action of Nanomaterials, namely*:*

- Composition, which includes inherent molecular toxicity, charge, hydrophobicity and coating (although also linked to both the intrinsic and extrinsic axes).
- *Intrinsic* properties, which are inherent to the nano-form of a material, and include e.g. structure and structural strain. A number of NM physicochemical properties map onto the intrinsic axis, including shape, porosity, structural configuration and bandgap.
- *Extrinsic* properties, which are those connected to the surface area of the NM, including e.g. surface interactions and transformations of NM surface and biomolecules (e.g. unfolding, receptor activation, membrane damage, fibrillation etc.) as a result of binding.

Clearly, each of these PCs/axes will have multiple contributors, and the relative contribution will vary for different NMs and will need to be teased out as part of the overall quantification of each PC. Key features of this approach are that: (1) it allows separation of modes of action (e.g. dissolution is primarily associated with specific NM *compositions*, but can be facilitated by specific *intrinsic* properties such as high strain conformation associated with, for example, pointed structures such as needles or nano-stars, and by *extrinsic* factors such as strongly binding ligands that unfold and expose cryptic epitopes for example; and (2) identities the major physico-chemical descriptors driving the toxicity from the weight of contribution to each PC/axes can be explicitly determined (i.e. the loadings), as can the main mode of action as this will be the PC accounting for the highest amount of the variability in the data, as shown schematically in Figure 2. Thus, we envisage utilisation of a set of scales from low to high (toxicity) for each of the three parameters with the overall toxicity being the sum of the three axes. The fact that the same physico-chemical descriptor can contribute to more than one PC is a key feature of this approach, which we expect will enable development of QNARs, facilitate the grouping of NMs on the basis of where they sit in this 3-dimensional space, and support regulatory decision-making. The role of *intrinsic* (structural) and *extrinsic* (surface and interface with media) properties has only recently begun to emerge in the context of nanotoxicity descriptors; the relative significance of these two groups of properties, as well as internal scaling are yet to be established.

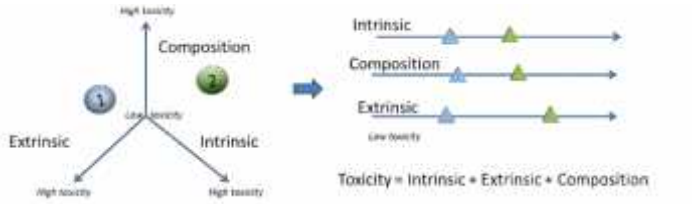

Figure 1. A principle components approach to predicting nanomaterial toxicity, where the experimentally determined physico-chemical parameters are mapped onto 3 axes (principle components), proposed here as composition, intrinsic properties (i.e. onherent to the nanostructure), and extrinsic properties (i.e. affected by the surroundings). By determining/predicting where a specific nanomaterial sits in terms of each scale it will be possible to predict its toxicity. From: I. Lynch, et al., Nano Today (2014).

Interest for industry & services: Grouping is currently the Holy Grail of nanosafety research. The expectation is that it will help tackle the overwhelming quantity and diversity of MNMs currently on and coming to the market and especially allow grouped rules for risk assessment and safety-by-design, saving a lot of effort, e.g. in the frame of REACH and in the development of new products.

Nanomaterials transformations in the environment

Partners EMPA and CEA have reviewed the potential and product-use related aging, alterations and transformations of nanomaterials (NMs) in the environment, focussing on identifying transformations that increase the similarity of NMs and those that decrease the similarity of NMs. The focus is on processes resulting in NM release and on the transformation(s) the released particles undergo in various phases of its life cycle for several nanomaterials (Ag, ZnO, TiO_2, carbon nanotubes, CeO, etc.). These include photochemical transformations, oxidation and reduction, dissolution, precipitation, adsorption and desorption, combustion, biotransformation, and abrasion among other biogeochemical processes.

To date, few studies have tried to establish what changes the ENPs undergo when they are incorporated into, and released from, products. As a result there is major uncertainty as to the state of many NMs following their release because much of current testing on pristine NMs may not be fully relevant for risk assessment purposes. The goal of the review was therefore to use life cycle thinking to derive possible transformations common ENPs in nano-products may undergo based on how these products will be used by the consumer. By determining specific gaps in knowledge of the ENP transformation process, this approach should prove useful in narrowing the number of physical experiments that need to be conducted and illuminate where more focused effort can be placed.

This work was presented (along with other NanoMILE outcomes, and those from NanoFATE, ModNanoTox, QualityNano and other EU FP7 projects at the OECD Classification wortshop in Washington DC in Spetember 2014.

See Mitrano et al., Environ Int. 2015.

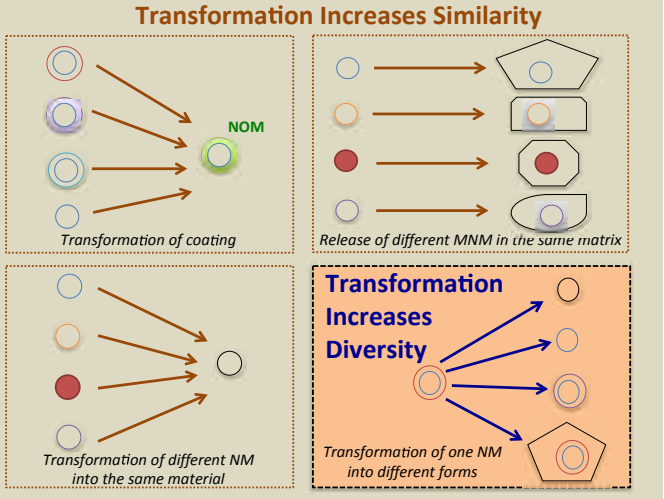

Figure 2: Transformation reactions can make different NMs more similar (coating of various forms with NOM; transformation into same phase, e.g. of Ag-forms into Ag_2S; release of different NM inside the same matrix) which may determine whether their fate and exposure scenarios would be simplified. Conversely, transformations may increase MNM diversity, where different transformations result in various forms of one NM-type.

Interest for industry & services: The knowledge of the fate of MNMs in the environment is essential for a relevant and efficient risk assessment, allowing efforts to be focused in the right place. The protocols established could be proposed by laboratories, such as those already proposing leakage tests for soils and waste.

Cellular self-organisation and mobility on micro-structured surfaces: towards single cell HTS

Partner LMU has been working on methods to pave the way for automated filling of cell arrays, enabling high-throughput single-cell analysis of cell samples without losses. LMU reported recently on the phenomenon of cellular self-organization, which allows for autonomous positioning of cells on micro-patterned surfaces over time. This was facilitated by preparing surface with a regular lattice of protein-coated adhesion sites surrounded by polymer (PLL-g-PEG) passivated areas, which reduce protein binding and thus cellular adhesion, and studying the time course of cell ordering. After seeding, cells randomly migrated over the passivated surface until they find and permanently attach to adhesion sites with occupancy levels typically reaching 40-60% after 3-5 h. The time required for sorting was found to increase with increasing distance between adhesion sites, and is well described by the time-to-capture in a random-search model. Further studies assessed cell behaviour (mobility) on polymer surfaces of different composition incubated with fibronectin (FN) using time-lapse microscopy, and correlated adhesion with the amount and location of FN via neutron reflectivity. Cells exhibited 21% increased motility on PLLPEG (5 kDa PEG chains) compared to pure FN layers, and 12% decreased motility for PLLPEG (2 kDa PEG chains), suggesting that by design of PEGylated surfaces cell migration can be controlled.

See Röttgermann et al., *Soft Matter.* 2014.

Interest for industry & services: High Troughput (HT) techniques allow the toxicity of different solutions on many cells to be tested simultaneously. They have replaced long, tedious and expensive manual work in hazard assessment. The disposition of cells according to a regular geometric pattern would allow high-throughput single-cell analysis of cell samples without losses, thus suppressing random effects, and reducing the number of experiments for the same level of significance. Producers of HT equipment could develop devices to benefit from this effect.

Comparison of 5-day and 28-day inhalation toxicity responses to ceria nanomaterials

Two ceria nanomaterials (NM-211, NM-212) were tested for inhalation toxicity and organ burdens in order to design a chronic and carcinogenicity inhalation study (OECD TG No. 453). Rats inhaled aerosol concentrations of 0.5, 5, and 25 mg/m³ by whole-body exposure for 6 h/d on 5 consecutive days for 1 or 4 weeks with a post-exposure period of 24 or 129 days, respectively. Lungs were examined in bronchoalveolar lavage fluid (BALF) and histopathology.

Inhaled ceria NM-212 was deposited in the lung and cleared with a half-time of 40 days; at aerosol concentrations higher than 0.5 mg/m³, this clearance was impaired resulting in a half-time above 200 days (25 mg/m³). After 5 days of exposure to concentrations >0.5 mg/m³ ceria induced an early inflammatory reaction by increases of neutrophils in the lung which decreased after the exposure was terminated. The neutrophil number observed in bronchoalveolar lavage fluid BALF was also decreased after 4 weeks of exposure (compared to 1 weeks of exposure) and an increase of mononuclear cells, especially macrophages was observed. Macrophages were visible in histopathology but not in BALF. Further progression to granulomatous inflammation was observed after 4 weeks plus 4 weeks post-exposure. The surface area of the two ceria nanomaterials (NM-211, NM-212) deposited in the lung, rather than mass or volume, correlated with the inflammatory reaction after 1 week of exposure. Observing the time course of lung burden and inflammation, it appears that the dose rate of particle deposition drove an initial inflammatory reaction by neutrophils. The later phase (after 4 weeks) was dominated by mononuclear cells, especially macrophages. The progression toward the subsequent granulomatous reaction was driven by the duration and amount of the particles in the lung. The further progression of the biological response will be determined in the ongoing long-term study.

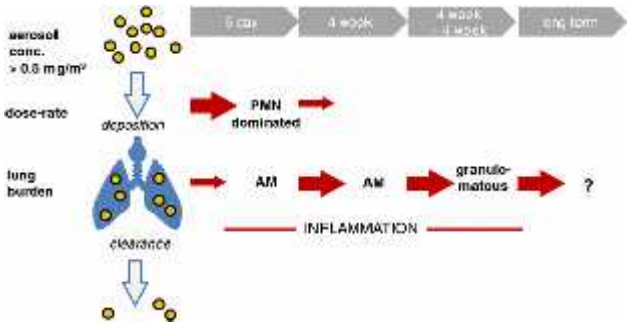

Figure 3: Summary of results after 5 days and 4 weeks of inhalation exposure to Ceria nanomaterials

See also: Keller et al. ***Arch Toxicol.*** 2014.

Interest for industry & services: The results of this experiment based on an OECD Technical Guidance will be directly applicable for the regulatory assessment of the MNMs concerned, e.g. REACH regulation or the development of Occupational Exposure Levels (OELs) for workers and of Toxicological Reference Values used in risk assessment for non-workers.

New tools to assess nanoparticle protein corona

Nanoparticles (NP), when exposed to biological fluids, are coated by specific proteins that form the so-called protein corona. While some adsorbing proteins exchange with the surroundings on a short time scale, described as a "dynamic" corona, others with higher affinity and long-lived interaction with the NP surface form a "hard" corona (HC), which is believed to mediate NP interaction with cellular machineries. In-depth NP protein corona characterization is therefore a necessary step in understanding the relationship between surface layer structure and biological outcomes.

UCD have been developing methods to evaluate the protein composition and stability over time, by systematically challenging the formed complexes with surfactants to denature the proteins and/or remove them from the corona. Composition and density of HC together with size and ζ-potential of NP–HC complexes were tracked at each step after surfactant titration. Results on Si NP–HC complexes showed that Natriumdodecylsulfat (SDS) removes most of the HC, while dodecyltrimethylammonium bromide (DTAB) induces NP agglomeration. Analogous results were obtained for PS NP–HC complexes. Interestingly, CHAPS and Triton X-100, thanks to similar surface binding preferences, enable selective extraction of apolipoprotein AI (ApoAI) from Si NP hard coronas, leaving unaltered the dispersion physicochemical properties. These findings indicate that surfactant titration can enable the study of NP–HC stability through surfactant variation and also selective separation of certain proteins from the HC.

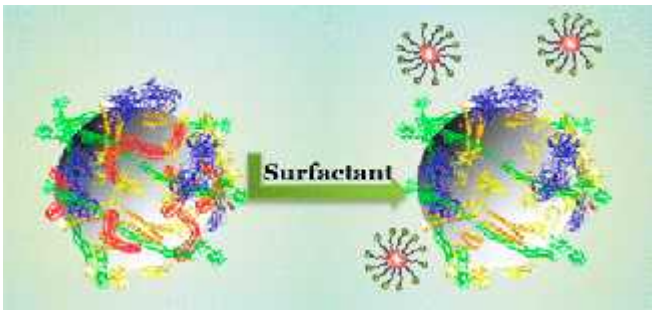

Figure 4: Schematic illustatration of the use of surfactants to « challenge » the nanoparticle protein corona. Impacts from surfacatnt include slective removal of specific proteins resulting in altered compostion of the corona, or induction of NM agglomeration.

See: Maiolo et al., *Anal Chem.* **2014** 86(24):12055-63.

Interest for industry & services: This approach has an immediate analytical value as well as potential applications in HC engineering.

Impact of NM redox activity on *in vitro* and *in vivo* endpoints

NanoMILE developed a set of ceO particles with different redox activities (achieved by doping with different amounts of zirconium oxide) by PROM. By increasing the amount of Zirconium, the resulting particles had less Ce^{4+} (and more Ce^{3+}) on the surface of the nanoparticles, and thus have less affinity for electrons and thus less capacity to scavenge radicals resulting in more oxidative stress and thus increased toxicity. Thus, the main in vivo study is assessing the influence of the redox activity of CeO_2 nanoparticles on the biological mechanisms behind the toxicological (respiratory, immunological, cardiovascular, neurological and allergic) effects after 28-days of exposure by inhalation, as shown schematically in Figure 7 below. The full set of exposures have been performed and data analsysis is nearing completion and will be published in a series of papers shortly, with contributions from RIVM, IUF, UNIEDIN, UoB and PROM in the near future.

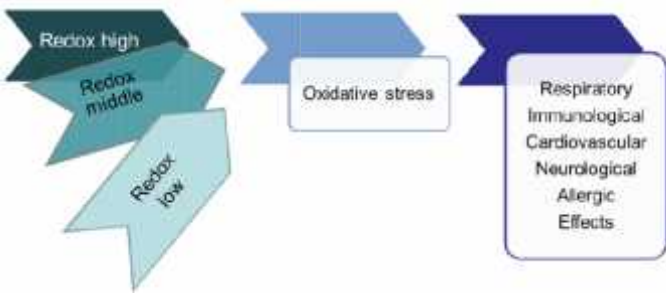

Figure 5. Schematic of the use of CeO_2 nanoparticles with different degrees of redox activity to probe the biological mechanisms behind the toxicological effects after 28-days of exposure by inhalation.

Interest for industry & services: These studies will open safer-design of CeO2 MNMs through Zirconium addition, and possibly also for other MNMs through modification of the redox activity.

Enalos InSilicoNano platform for QSARs

In an effort to computationally explore available datasets that will lead to ready-to-use applications one of NanoMILE's SME partners (Novamechanics) has developed and validated a quantitative nanoparticle-activity relationship (QNAR) model for the prediction of the cellular uptake of nanoparticles in pancreatic cancer cells. Their insilico workflow was made available online through the Enalos InSilicoNano platform (http://enalos.insilicotox.com/QNAR_PaCa2/), a web service based solely on open source and freely available software that was developed with the purpose of making our model available to the interested user wishing to generate evidence on potential biological effects in the decision making framework. This web service will facilitate the computer aided nanoparticle design as it can serve as a source of activity prediction for novel nano-structures. To demonstrate the usefulness of the web service we have exploited the whole PubChem database within a virtual screening framework and then used the Enalos InSilicoNano platform to identify novel potent nanoparticles from a prioritized list of compounds.

The EnalosInSilicoNano platform has a user friendly interface with minimum steps required and no authentication and authorization procedure. To initiate a prediction the user must first select the model of interest from the drop down menu provided, for which decision some guidance documentation and a decision tree are being developed. When the model "QNAR_PaCa2" is selected the prediction can be initiated when a structure or a batch of structures is uploaded. For that the web service provides three different options described as follows: (i) the user draws a chemical structure of interest using the drawing tool. The user can easily select from the different panels the atoms, bonds or substructures of interest and construct the molecule. What is important is that the user can also open, save and convert files with a variety of chemical formats (i.e. SMILES, IUPAC chemical Identifier, MDL MOL file) using the drop down menu of the online sketcher; (ii) the user enters the SMILES notation of a structure or several structures separated by newlines. Even if the SMILES notation is not initially known it is important that the chemical sketcher included gives the users the opportunity to design the chemical structure and then copy the structure as SMILES from the Edit drop down menu. This is very significant as it facilitates the generation of several structures since the user can make several modifications using the sketcher and copy all structures as SMILES so that a prediction for the whole set of produced structures is generated. The user can thus visualize the modifications and make multiple predictions at once; (iii) the user can select and import an SDF file (.sdf) with several structures.

When structures are uploaded in either way a prediction can be generated by clicking the submit button. The output is then presented in a different html page. The results include the predicted value for each structure entered and an indication of whether the prediction could be considered reliable based on the domain of applicability of the model.

See Melagraki and Afantitis, ***RSC Adv.***, 2014, **4**:50713.

Interest for industry & services: This tool can be used directly by MNM developers to test variants of potential new MNMs and pre-select the safer ones for further development.

Automated Exposure Station at the air/liquid interface

In the frame of NanoMILE, the German SME and NanoMILE partner Vitrocell developed an Automated Exposure Station which simulates the direct exposure of biological test systems to airborne substances such as gases, complex mixtures, nano particles and fibres. It offers a capacity of up to 18 cell culture compartments for exposure and 3 compartments for clean air control.

The system simulates the human exposure situation. All key functions for a successful exposure such as aerosol flow rates, humidity, temperature and leak test are edited via touch screen prior to the experiment. The respective data is shown on live graphs and stored for further analysis. The cells are exposed at the air / liquid interface on 6, 12 or 24 well sized cell culture inserts. The isokinetic sampling system enables a uniform delivery of the test substance to the cells. The deposition efficiency can be increased by high voltage charging.

After exposure the cells are further processed to measure a wide range of endpoints, e.g. cytotoxicity, genotoxicity, proliferation, cellular and oxidative stress as well as inflammation.

Figure 6: Vitrocell's Automated Exposure Station

The future action within the NanoMILE project will be to establlish a comprehensive and solid set of associated recommended methods (from cell cultivation to interpretation), based on collaboration between NanoMILE partners Vitrocell, KIT and RIVM (SOP drafting, comparison of results…).

See on Vitrocell's website: http://www.vitrocell.com/inhalation-toxicology/exposure-systems/automated-exposure-station.

Interest for industry & services: This automated station will allow more cost-effective testing of cell-toxicity for inhalation.

NanoMILE's publications to date:

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