NanoMILE

Engineered nanomaterial mechanisms of interactions with living systems and the environment: a universal framework for safe nanotechnology

Contract Agreement: NMP4-2012-Large-310451Website: http://www.nanomile.eu
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3	University College Dublin, National University of Ireland, Dublin	NUID UCD	Ireland
4	Commissariat a l'Energie Atomique et aux Energies Alternatives	CEA	France
5	Joint Research Centre of the European Commission	JRC	Belgium
6	Eidgenössische Materialprüfungs- und Forschungsanstalt	EMPA	Germany
7	Eidgenössische Anstalt für Wasserversorgung, Abwasserreinigung und Gewässerschutz	EAWAG	Switzerland
8	University of Geneva	UoGEN	Switzerland
9	Rijiksinstituut voor Volksgezondheid en Milieu / Institute for Public Health and the Environment	RIVM	Netherlands
10	The University of Exeter	UNEXE	United Kingdom
11	Ludwig-Maximilians Universität, München	LMU	Germany
12	The Regents of the University of California	UCLA	United States
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19	Novamechanics Ltd.	NM	Cyprus
20	Nano4imaging GMBH	N4I	Germany
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22	Promethean Particles Ltd.	PROM	United Kingdom
23	Eurofins Agroscience Services GMBH	EF	Germany
24	European Virtual Institute for Integrated Risk Management	EUVRI	Germany
25	BASF SE	BASF	Germany
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1 Summary

Project Duration: 48 months

Project Funding: 10 M€

Nanotechnology is a rapidly evolving enabling technology with the potential to revolutionise modern life. On the nanoscale, common materials can take on entirely new chemical, physical and biological properties. These properties open up new possibilities for exploitation and commercial enterprise. However, an increasing body of scientific evidence would suggest that some materials in their nano-form may induce harmful biological or environmental effects through a variety of potential mechanisms, not all of which are fully understood or quantified as yet. Such questions are addressed by the rapidly expanding field of "nanosafety". Indeed, although significant research efforts have been made to make the risk assessment of nanotechnology possible, we are still lacking a mechanistic and systematic understanding of which physico-chemical parameters, or combination of parameters, govern the toxicity of nano-sized objects. Thus, we remain unable to ensure the protection of health and the sustainable commercialisation of nanotechnology.

NanoMILE intends to revolutionise nanosafety research through its robust and novel approaches to the selection and development of the test nanomaterials, its technically and computationally advanced integration of systems biology, its thoughtfully balanced toxicological / ecotoxicological approaches, its development of novel high throughput platforms for screening and its feedback loops for development of nanomaterials that are safer by design. Together, these approaches will result in ***a robust framework for classification of nanomaterials according to their biological impacts***. The advanced scientific expertise offered by the academic partners has been matched by a complement of fully committed and well integrated industrial partners, capable of contributing to or advancing the innovations of NanoMILE to industrial applications.

The NanoMILE project commenced on 1st March 2013 and will run for 48 months.

2 Background

NanoMILE builds on several highly successful previous FP7 projects lead by the coordinator, specifically NanoReTox and ModNanoTox. In particular, NanoReTox developed an approach to normalize data across the concentration ranges utilized for *in vitro* and *in vivo* studies and developed a heat-map approach to categorizing nanomaterials according to their toxicity. A key finding from NanoReTox was that intrinsic nanomaterial composition is the primary driver of toxicity, with copper oxide being the most toxic from the panel of metal oxide and metal particles assessed within that project. Building on that knowledge, NanoMILE has made particle choices that include both known benign materials, that we will attempt to make toxic by altering their properties in a systematic manner, and known toxic nanomaterials that we will attempt to make safer by systematically varying their properties, in order both to isolate (and derive a threshold value) for the various drivers of toxicity, and to develop a set of rules for safer by design nanomaterials. The systematically varied libraries of nanomaterials developed in NanoMILE will also form the basis for high content screening approaches and on the basis of the outcomes from the screening, for detailed assessment of toxicity and ecotoxicity across a range of end-points and species, in order to identify commonalities in terms of mechanisms of action.

From the ModNanoTox project, which was one of the EU-US modeling projects, focused on development of models for assessment of the environmental impact of nanomaterials, NanoMILE builds on the experiences regarding the limitations of modelling approaches, where, for example, Lazar approaches were demonstrated to be inapplicable for nanomaterials in an

environmental context due to significant data gaps that resulted in the training data set being insufficiently offset from the test data set for reliable correlations to be achieved (a public report on the outcomes and challenges is available for the NanoSafety Cluster). Thus, there are also some specific particle requirements for the QSAR modelling and the data integration that have been factored into the development of the nanomaterials libraries for NanoMILE.

3 Scientific and Technical Challenges

Despite being relatively new, nanoscience and nanotechnology have advanced rapidly in terms of generating scientific discoveries along with commercial applications. However, the field of nanosafety, which is the science of assessing hazards and risks from novel nanomaterials, has not kept pace with these developments and relevant to this project are some key areas where the current state of the art requires urgent progression and advancement in understanding. Potentially the greatest concern in the science of nanosafety is the lack of a paradigm for MNM mode of action, as emphasized in the recently published report by OECD Sponsorship Programme for the Testing of Manufactured Nanomaterials, which necessitates that each MNM is considered individually for its toxicity.

Here we highlight some key shortfalls and gaps in knowledge regarding nanosafety and illustrate how the NanoMILE project will address these and ultimately provide a new paradigm in nanosafety, thus substantially advancing the field beyond the current state of the art.

Challenge 1: *A large number of MNMs exist, many already in industrial production. Often behaviour and toxicity of nominally identical MNMs vary, perhaps a result of poor characterisation or understanding of their structure and complexity or perhaps resulting from batch-to-batch differences or poor synthesis control. Studies of the effect of a systematic variation in properties of MNMs on biological reactivity including toxicity are virtually non-existent. A paradigm systematically linking MNM properties with biological effects / toxicity is urgently needed.*

NanoMILE will select, synthesize/procure MNMs suitable for hypothesis-driven development of mechanistic models of nanomaterial interactions with organisms and the environment. To advance the current state of the art, it is essential to include in our study material MNMs designed to display systematic property variations, so that prototypic mechanisms of action of MNMs can be linked directly to specific properties and input into QS(P)AR models. Far from allowing these "designer" MNMs become obsolete at the end of the project, NanoMILE will redesign these MNMs in WP9 to make them safer by design.

Challenge 2: *Many MNMs are likely to undergo significant transformations during their life cycle, following their release and as they move into different biological or environmental compartments. These transformations have received limited attention to date & predictions of MNM behaviour are unsupported by robust data.*

NanoMILE will investigate and quantify the alteration and transformation of MNMs in products and during their use and release into the environment or biota. Exposure to MNMs in occupational, consumer or environmental settings may either be to the original, parent MNMs or to MNMs that have been incorporated into products and subsequently released, either in their original or altered form due to industrial or natural processes.

Challenge 3: *There are simply too many different MNMs to be tested by any one project or lab. Harmonisation of data across labs is a further challenge. A high throughput platform for hazard ranking is required to address this.*

One of NanoMILE's pioneering approaches is the practical incorporation of a high throughput platform, which will allow screening of a large numbers of MNMs/MNM variants at the start of the project, in order to identify "lead candidates" for subsequent work. High throughput and content screening (HT/CS) *in vitro* (cell culture) and *in vivo* (zebrafish) will therefore be established. The same high-throughput approach will be used again later on for the validation of results and establishment of causality of the discovered biomarkers for subsequent toxicity by using chemical and genetic interference strategies. The large volume of data generated by this work will be instrumental for the quantitative structure (property)-activity relationships (QS(P)ARs), to allow identification of no-observed-adverse-effect levels (NOAELs) and to predict the impacts from physico-chemical characteristics or "initial" corona characteristics. Notably, latter aspects of these innovations will be advanced to demonstration stage by industrial partners.

Challenge 4: *MNMs transform upon contact with biological or environmental media, and it is likely that a layer of biomolecules or geomolecules ("corona") cover their surface. The nature, properties and robustness of this layer and interactions between the core and the corona are currently poorly understood; it is also not clear how different environmental or biological compartments will impact on the formation of this corona.*

Beyond the current state of the art, NanoMILE will focus on the quantification (which has not been addressed to date) of MNMs interactions with environmental and biological macromolecules (proteins, lipids, sugars, nucleic acids, humics) before and after uptake and localisation, and correlation of nanomaterial-associated biomolecules with

nanomaterial fate and behaviour in cells, organisms and animals. An important and novel objective will be to establish the precise nature and transformations of the coronas with time in realistic environmental conditions. Modelling of NP-biomolecule interactions will be included and data will feed into the development of QS(P)ARs. Methods will be optimised to be applicable for identification and quantification of proteins, lipids, sugars, natural organic matter etc., associated with nanomaterials over timescales of relevance for biological interactions (minutes) and each of the tasks will be conducted for a range of different biofluids, representative of the different exposure routes (inhalation, ingestion, intravenous, environmental (e.g. aquatic/terrestrial).

Challenge 5: *Although toxicological studies exist for a number of different species, many such studies produce different results and there is no framework for comparisons across species and in different environmental compartments (terrestrial / marine / freshwater). It is becoming clear that nanoparticles react with biota in a specific manner where toxicity is one of the outcomes of these interactions. Others may include reduced energy reserves, reduced fitness and ultimately increased vulnerability.*

NanoMILE will carry out investigations into in vivo bioavailability and effects related to nanoparticle exposure across wildlife species from single celled organisms to lower vertebrates (fish) and from subcellular to ecosystem level thus creating one coherent set of parameters for multiple species and MNMs. We will test hypotheses that specific features of MNMs confer toxicity through the use and application of modified MNMs and identify common effects across a wide range of wildlife taxa and establishing the most vulnerable organisms for potential harm. The focus will be on algae, daphnia, aquatic isopods and worms, and fish (zebrafish: adults and embryos), and for terrestrial animals *Caenorhabditis elegans*, earthworms (*Eisenia fetida*), springtail (*Folsomia candida*), and soil mite (*Hypoaspis aculeifer*) and a range of isopods with varying ecological niches. ENP selection will be based on results from the high throughput testing (WP4). This is an extensive set of organisms and MNMs tested under a universal framework and will generate a unique and valuable database.

Challenge 6: *Although a substantial volume of mammalian toxicological studies exist (in vivo and in vitro) a model for human toxicity has not yet emerged.*

NanoMILE will evaluate distribution (biokinetics) and toxicological endpoints after exposure of cells, isolated organs and organisms. Nanoparticles with defined composition, size distribution, and surface properties from WP2 will be transferred into an aerosol with defined size/morphology, and deposited on lung cells via the air/liquid interface with well defined mass, number, and surface doses. For other cell types, submerged systems will be used. Mechanisms of toxicity (e.g. oxidative stress, inflammation, thrombogenicity) indicative for the induction of clinical adverse effects will be identified and correlated over the various physico-chemical characteristics and test systems in the project. There will be a focus on inhalation toxicity studies using aerosols, as this is one of the most likely exposure routes for humans, but both oral and intravenous application will also be used as relevant routes of exposure. Migration of MNMs, physical stress including frustrated phagocytosis and more complex responses of the immune, cardiovascular or central nervous system might be predicted using novel cell based *in vitro* systems as applied in this project.

Challenge 7: *Systems biology has in recent years emerged as a powerful tool for understanding biological mechanisms at the molecular level and using such information to generate predictive and mechanistic approaches in disease. These advances have yet to be applied in the field of nanosafety.*

NanoMILE will seek to discover and compare mechanisms and potencies of the potential harmful effects of different MNMs using an integrated Systems Biology approach, including transcriptomics, metabolomics, lipidomics and computational biology. These consortium participants are highly experienced in the application of 'omics technologies to studying biological responses to toxicants. The overall aim is to identify prototypic mechanisms of action of MNMs, including both species-specific and evolutionarily conserved responses, with the latter likely to provide extremely powerful biomarkers in relation to assessing MNMs impacts on environmental and human health. This WP is linked tightly with high throughput work (WP4), both in regard to the initial selection of MNMs for detailed analysis and the application of the discovered novel molecular biomarkers in subsequent high throughput screening (HTS).

NanoMILE will employ both static and dynamic modelling to identify subsets of the multi-dimensional, information rich, 'omics datasets that represent adverse outcome pathways (AOPs), i.e. mechanistically based molecular biomarker signatures that can be implemented into diagnostic screening assays to identify and characterise the impacts of nanomaterials. So-called "Reverse Engineering" approaches, which are a branch of Systems Biology, will be used to reconstruct the underlying structure of biological pathways from observational 'omics data. The dynamical models will also enable *in silico* simulations of the toxicity responses to MNMs, which will be tested experimentally.

Challenge 8: *No platform exists for referencing and comparing the activity, in terms of toxic behaviour, of MNMs; no fundamental concept of safe MNM design has yet been developed.*

Following early work within NanoMILE which will discover systematically the precise mode of action of MNMs properties, key later activities will be carried out towards:

a) practically test such features by designing them in or out (both at bench and pilot scale);

b) develop models of quantitative structure (property) – activity relationships (QS(P)ARs) enabling predictive work to evolve and feed into risk assessment; and

c) provide an integrated platform for risk assessment.

In order to design safer MNMs, the work in NanoMILE will involve a central iterative link between MNM properties and biological/environmental effects, i.e. if certain features of the particles become clear as inducing toxicological effects, then these features will be designed out in WP9 (keeping all other parameters constant as far as possible) and the particles will be re-tested to confirm those features conferred the observed toxicity; the opposite (design in features to create positive controls of certain magnitude) will also be applied. Once these modifications are tested and the principles of safer designs are established for one group of MNMs, similar principles will be transposed to other families of MNMs, to establish whether these apply and whether generic patterns of safer designs may begin to emerge.

One of the ultimate goals will be to test if this approach works across structurally and chemically different MNMs and across a range of sizes. Carbon based materials form a separate class of materials, although similarities in issues related to surface modifications apply across all classes. Designing safer MNMs will then be implemented at demonstration level by industry partners.

Challenge 9: *A lot of projects operate in isolation both laterally by not interacting with other concurrent research on the same or similar topic and temporally by missing existing background and allowing the generated foreground to lapse after the project ends.*

NanoMILE has a WP & team ensuring interactions with other major funded projects, to ensure recently acquired state of the art flows smoothly into the project, parallel developments from ongoing work are known to the research teams and future developments through NanoMILE flow into other projects and applications, ensuring the maximum possible impact to emerge from the project.

4 Project Objectives

The overarching objective of NanoMILE is to formulate an intelligent and powerful paradigm for the mode(s) of interaction between manufactured nanomaterials (MNMs) and organisms or the environment to allow the development of a single framework for classification of nanomaterial based on their potential toxicity and to create a universally applicable framework for nanosafety.

Specific objectives, in chronological order of development, are:

• **Objective 1:** To select and synthesise/procure MNM libraries suitable for hypothesis-driven development of mechanistic models of nanomaterial interactions with organisms and the environment, in harmony with, and linking to existing EU funded platforms, such as the EU funded QNano or the sponsorship programme of the OECD Working Party on Manufactured Nanomaterials (WP2).

• **Objective 2:** To establish an understanding of changes in the nature of MNMs as they undergo transformations within products and biological or environmental compartments across their life cycle and critically to feed this information into subsequent research to ensure that these "aged" and transformed MNMs are tested for their biological/environmental role (WP3).

• **Objective 3:** To establish a screening platform (WP4) based on high throughput techniques at two stages: a) at the start of the project, to screen for the most relevant MNMs and endpoints (using both classical and novel biomarkers) to provide a focus for subsequent WPs (5-8) and later, b) to screen the mechanistic discoveries from WP5-8 and develop test methods of the future.

• **Objective 4:** To qualify and quantify nanomaterial interactions with environmental (humic acids, polysaccharides, clays) and biological molecules (proteins, lipids, sugars, nucleic acids) before and after uptake into biological systems to enable understanding of how these interactions alter MNM fate and behaviour in cells, organisms and animals. To generate a computational-based screening platform for bionano interactions to allow tests on a comprehensive dataset of MNMs (WP5).

• **Objective 5:** To establish in-vitro and in-vivo reactions between MNMs and a carefully selected range of cell-lines/organs/organisms, representative of a wide range of species with increasing biological complexity, from algae to fish, aquatic and terrestrial species (WP6) and humans (WP7).

• **Objective 6:** To complement the above with a carefully selected range of systems biology based studies (WP8) to support the understanding and comparisons of mechanisms of MNMs activity across several species of increasing complexity.

• **Objective 7:** To more intelligently design safer MNMs (WP9), using the previous WPs as a guide, and working towards designing out adverse effect causing features.

• **Objective 8:** To develop appropriate models linking quantitative structure (property)-activity relationships (QS(P)AR), established from the biological effects studies above, to population response models, thus enabling predictive work to evolve from molecular mechanisms (specific toxicity pathways **and classification of MNMs according to their mode of action) to the scale of the ecosystem (WP9).**

• **Objective 9: To interact closely with other EU and US funded projects and the NanoSafety Cluster, to ensure maximum integration of prior state of the art within the project and progression along and beyond paths and platforms thoughtfully designed by these projects (WP10).**

The Workpackages (WPs) listed in the text above are interlinked and in constant communication with feedback-loops where information is iteratively fed into the WPs as shown in Figure 1.

The scientific and technical goals of NanoMILE, as outlined in Section 2 above, could not be achieved by an effort at a national level. All the project partners are leaders in their respective fields, and have truly complementary scientific skills. None of the European states involved would individually have access to such a pool of competencies. This also applies to the range of facilities and resources mobilised by NanoMILE.

The NanoMILE consortium comprises 28 partner organisations selected for their ability to play unique and essential roles in the consortium. Of the 28 organisations, 10 are universities, 3 are research facilities, 5 are government bodies, 2 are multinational companies and 8 SMEs (3 technical consultants, 4 materials/instrumentation manufacturers). The two US partners are critically selected and ideally placed to add strength to the consortium by providing expertise at the highest technical level, thus matching and augmenting the capabilities of the European part of the consortium.

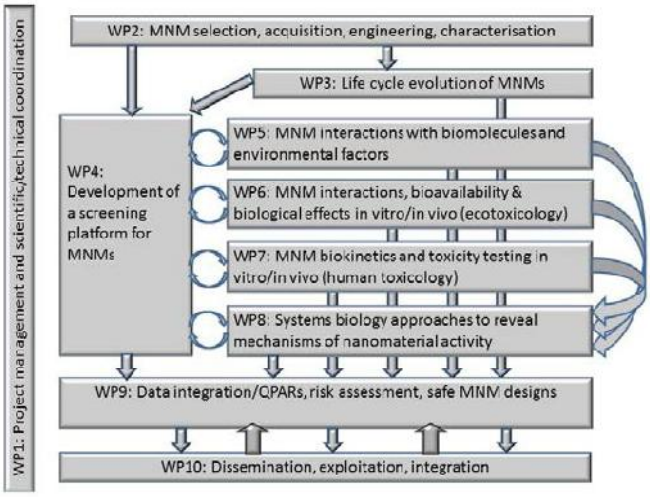


Figure 1. NanoMILE WP flow diagram & interdependencies

5 Progress and Outcomes to date

NanoMILE reached and successfully passed the M24 (mid-term) milestone, with a successful project meeting in Brussels on 22-24 April 2015. All workpackages are progressing well, with a range of exciting outputs being presented at numerous key events, such as the NanoTox2014 meeting in Antalya, Turkey, the SENN2015 meeting in Helsinki in April 2015 and others, as well as numerous activities planned around NanoMILE's booth at the EuroNanoForum in Riga (June 2015) and abstracts submitted for the ICEENN 2015 conference in Vienna in September 2015.

Numerous technical deliverables, including reports on the Phase 1 particle library, the approaches to ageing of nanomaterials, approaches for time-resolved characterization of nanomaterials and identification of body target tissues accumulating MNMs have been delivered to the Commission and publications are in preparation. Summaries of some of the key outcomes from the period June 2014-May 2015 are reported below. Interested readers are referred to the 2014 edition of the NSC Compendium for summaries of outcomes from the early phase of the NanoMILE project, including the nanomaterials library and the properties selected for systematic variation; and the initial classification/ grouping strategy developed within NanoMILE.

NanoMILE partners have been active in terms of disseminating their activities within NanoMILE from the outset of the project, with 26 individual dissemination activities being reported for first period (months 1-18), and an additional 11 in the period up to May 2015, as indicated in Figure 2. Indeed, the consortium has produced 12 published articles and 2 book chapters to date with 2 more submitted, as well as contributing three articles to the NanoSafety Cluster Newsletter, presenting talks at 7 international

conferences, including the EU-US CoRs meeting in the US (3rd U.S.-EU Bridging NanoEHS Research Efforts workshop. Arlington, VA, USA. 2-3/12/2013) and 2 invited talks at the NanoTox2014 conference in Antalya. 10 partners have been active in dissemination of NanoMILE specific activities to date, as indicated in Figure 3. Of the 14 publications to date, 3 are co-authored by two partner institutes and 3 are single-institute publications, as indicated in the list of publications below. A summary of the importance and key outcomes from selected publications is also provided below.

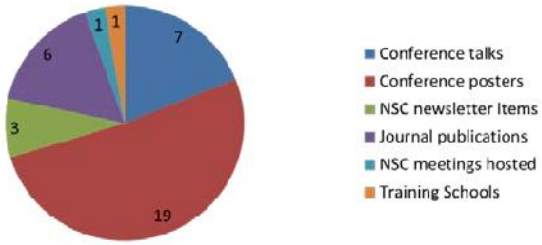
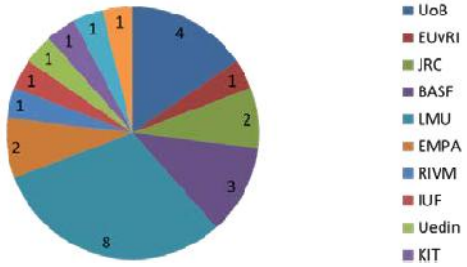


Figure 2: Summary of the dissemination activities to date.

Figure 3: Partners involved in dissemination activities.



Training Courses

NanoMILE had an opportunity to host / jointly-organise a hands on lab training course and workshop over 3.5-days with NanoFate and QualityNano, in March 2014. This was the first (only) hands-on lab course organised by QualityNano and was facilitated enormously by the collaboration with NanoFATE and NanoMILE, specifically in terms of the session on *Mechanistic toxicology of Engineered Nanoparticles (ENPs): state of the art, future perspectives and practical workshop on high-throughput molecular data* and the associated training session.

The three hands-on trainng sessions (each 1-day) delivered were:

- **Course 1:** Mechanistic toxicology and systems biology approaches and data analysis:
- **Course 2:** Making your own Gold NPs and characterising them and other NPs:
- **Course 3:** Environmental realism in NP dosing and experiments (practical considerations and modelling).

Speakers / trainers from NanoMILE included Damjana Drobne (University of Ljubljana) who spoke about Tracking internal distribution and effects of nanoparticles within organisms; Iseult Lynch (University of Birmingham) who spoke about Bionanointerfaces and the ecological corona – interactions, displacement and evolution and Eva Valsami-Jones (University of Birmingham) who spoke about significant trends in the physiochemical control of nanoparticle toxicity.

Highlights of key outputs/publications (specifically those not already covered in 2014 Edition of the Compendium):

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

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_2, 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.

Note that 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.

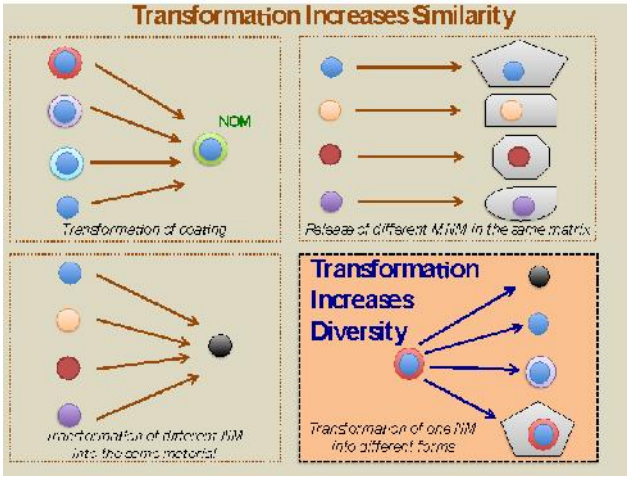


Figure 4: Transformation reactions can make different NMs more similar (coating of various forms with NOM; transformation into same phase, e.g. of Ag-forms into Ag_2S; release of different NM inside the same matrix) which may determine whether their fate and exposure scenarios would be simplified. Conversely, transformations may increase MNM diversity, where different transformations result in various forms of one NM-type from a specific source/product.

See Mitrano et al., Environ Int. 2015 Apr;77:132-47. doi: 10.1016/j.envint.2015.01.013

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 Apr 14;10(14):2397-404. doi: 10.1039/c3sm52419a

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 (Figure 5). 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.

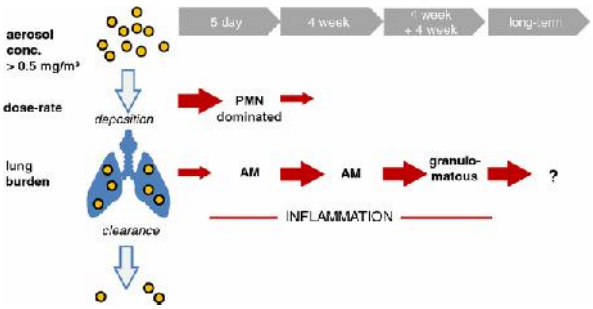


Figure 5: Summary of results after 5 days and 4 weeks of inhalation exposure to ceria nanomaterials

See also: Keller et al. ***Arch Toxicol.*** 2014 88:2033-59.

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 SDS removes most of the HC, while 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. This approach thus has an immediate analytical value as well as potential applications in HC engineering.

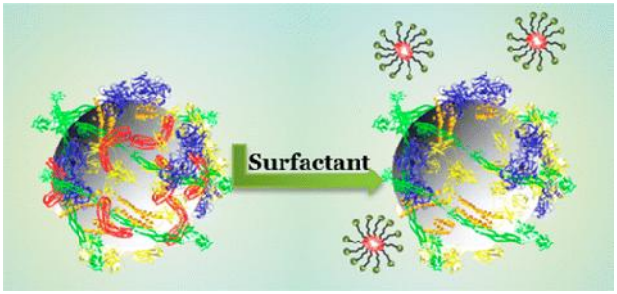


Figure 6: Schematic illustatration of the use of surfactants to « challenge » the nanoparticle protein corona. Impacts from surfacatnt include slective removal of specific proteins resulting in altered compostion of the corona, or induction of NM agglomeration.

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

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

Close collaboration bwteen WP7 and WP2 resulted in the development of a set of 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, with contributions from RIVM, IUF, UNIEDIN, UoB and PROM in the near future.

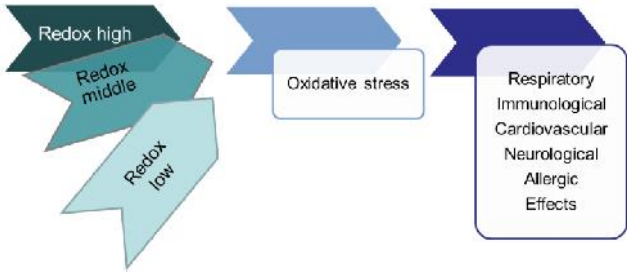


Figure 7. Schematic of the use of CeO_2 nanoparticles with different degrees of redox activity to probe the biological mechanisms behind the toxicological (respiratory, immunological, cardiovascular, neurological and allergic) effects after 28-days of exposure by inhalation.

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 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.

Automated Exposure Station at the air/liquid interface

In the frame of NanoMILE, the German SME / 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, nanoparticles 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, 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 7: 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 *via* exposure), based on collaboration between NanoMILE partners Vitrocell, KIT and RIVM (SOP drafting, comparison of results etc.).

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

NanoMILE's Brussels series of workshops:

NanoMILE is organising a series of workshops at the University of Birmingham's Brussels office, conveniently located at 22-28 Avenue d'Auderghem / Oudergemselaan, B-1040 Brussels, in the European Commission district. Each event in the series of workshops, called the "Brussels nanosafety consensus series", will be jointly organised with one of the other large projets (NanoValid, MARINA and NanoSolutions) and will focus on a specific theme, with the goal of preparing a European scientific position paper to feed into ongoing activities such as the OECD activities on Manufactured nanomaterials.

The proposed topics, co-organiseing projects, and approximate timeframes for the first set of workshops are as follows:

- Methodologies for phys-chem / biophys-chem characterisation (NanoMILE and NanoValid) - 7th and 8th October 2015
- Data curation & tools for interrogation of data / risk assessment (NanoMILE and MARINA) – date tbc

- Alternative test methods – high throughput & omics approaches (NanoMILE and NanoSolutions) – May 2016

Outputs from each workshop will be:

- A White paper & associated summary publication
- Agreed terminology for the topic
- Agreed protocols and approaches
- Agreed understanding of current limitations of the methods / approaches in terms of their applicability to different NMs classes
- Plan for benchmarking activities and buy-in of relevant projects with capabilities to achieve this.

Registration for the first events opens early June 2015 and will be posted on the NanoMILE website and the EU NanoSafety Cluster website.

Data curation and knowledge management

NanoMILE is acting as an NDCI Stakeholder Liaisons for the data curation project led by the NCI and EU-US CoR on databases and ontologies. As part of this we are sharing NanoMILE's best practice with the community via contributions to a number of surveys of best practice over the space of about 1 year leading to a series of publications as part of the Nanotechnology Data Curation Initiative.

6 NanoMILE's Expected Impacts

"Nanotechnology businesses and organizations will restructure toward integration with other technologies, distributed production, continuing education, and forming consortia of complementary activities."

The volume of MNM production has led to significant concerns about the risks to human health and environmental impact as potential pollutants of considerable importance. Sustainable development of ENMs in industry requires the minimisation of these risks. The results of the NanoMILE project will be formulated into a number of tools to assist industry and regulators in identifying where specific safety assessments might be necessary, and as such close links with NanoFutures, and the relevant ETPs will be implemented. A priority will be to support both industry and public acceptance via development of scientific principles as the basis for improved regulation with clear and simple rules. Currently, there appears to be a lack of knowledge in the general public, although there is broad support for nanotechnology where knowledge exists; an improved general knowledge of hazard, risks and benefits is therefore essential.

NanoMILE will contribute significantly to the efforts to reduce the many uncertainties about the potential impact of MNMs on health and the environment, which is urgently needed for the development of a sound regulatory framework. It is crucial to learn what the parameters are that govern the toxicity of nano-sized objects and what the underlying mechanisms are for the sustainable development of MNMs. It is also important to note that regulatory uncertainly leading to delays in commercialisation is more costly to business than clear additional regulatory requirements. A sound regulatory framework has also been requested by the European Parliament which considered it particularly important to address MNMs explicitly within the scope of legislation on chemicals, food, waste, air and water, and worker protections.

The NanoMILE consortium have already identified a number of key outputs that will have significant impact for the various stakeholders involved in the nanosafety and nano-commercialisation question, including numerous potential candidates for standardization, as follows:

- Descriptors for grouping / classification of MNMs (including aged MNMs)
- Algorithms and predictive models (& the associated Standard Operating Procedures, SOPs)
- New High-throughput (HT) assays for screening MNM impacts (cell-based, cell free) (& the associated SOPs), including 2 industry-led demonstration models
- Data management tools to link physico-bio-impact data from point of generation to mining ability (& the associated SOPs)
- 'Omics' datasets for the 4 test species in response to systematic sets of MNMs
- Design rules for tailoring MNM impacts – novel MNMs as Reference Materials etc.
- Data on controlled human and organism exposure & comparison to models of increasing complexity (& the associated SOPs)
- Nanoparticle libraries (along with synthesis, functionalization, purification SOPs) and safety dossiers for SME partners on their MNMs for use in business to business marketing.

NanoMILE's Exploitation Strategy Seminar in October 2014 has further crystalized our next steps towards exploitation and our Standardisation planning is advancing in parallel.

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8 Directory

Table 2. Directory of people involved in the NanoMILE project

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