NanoMILE

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

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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, capato ble 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 Lazar modelling approaches were demonstrated to be inapplicable for nanomaterials in an environmental context at present 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 emphasised 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.

A smaller range of MNMs will be purpose-designed for the project to address specific needs, where, for example, systematic property changes need to be tested or where freshly produced particles are required (e.g. respiratory effects of free MNMs versus aggregates/agglomerates, redox sensitive MNMs) or special labels need to be introduced (e.g. stable isotopes). This approach will give NanoMILE powerful tools to advance the current state of the art, held in many cases by project partners. Purpose made MNMs will also allow a systematic investigation of the effect of size within critical relevant size range as well as the role of shape and the presence of inorganic and organic nanomaterial coatings.

Extensive testing of a great number of MNMs is only possible through the high throughput platform of NanoMILE (see below). All materials procured or developed within this work package will be subjected to extensive physicochemical characterization using state-of-the-art methods (imaging, compositional and structural, and following where possible established (e.g. through QualityNano) protocols, thus avoiding problems of unreliable cross referencing of experimental results. The MNM characterization data will be integrated into the database of nanoparticle information being developed by partner Biomax.

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.

To date, few studies have tried to establish the changes that MNMs undergo when incorporated into, and released from, products [2] MNMs in textiles, paints, and sunscreens have, to some extent, been studied [3]. It has been shown that MNMs released from these products may be altered considerably and change their physical and chemical properties compared with the original MNM. Furthermore, the transformations that take place may vary considerably between MNMs, with some metals, such as Ag will potentially transform to sulfides, whereas certain metal oxides such as TiO_2 will remain largely unchanged over relevant timescales. A whole range of other behaviours may also take place, for example dissolution, complete or partial for some metal/metal oxide MNMs, or stabilisation by natural organic matter (humics and biomacromolecules) or proteins (see also below). As a result there is major uncertainty as to the state of many MNMs following their release.

WP3 will expose relevant MNMs selected from the libraries of WP2 to different processes, different biophysicochemical conditions, in order to characterize the changes in the MNM, and either deliver altered MNMs or provide detailed protocols on how to induce these alterations, to alternative WPs. These altered MNMs will then be used alongside the parent particles in WP4-9. Predictive models will be developed that describe release of MNMs from products to the environment and qualitatively and quantitatively assess the changes of MNMs properties during these processes. Significant advancement of the current state of the art will be through the generation of libraries of modified (but stable) MNMs for testing in subsequent WPs and by incorporating the effect of ageing as a further descriptor in the project's QS(P)AR models.

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.*

Cell lines and zebrafish embryos were recently used successfully for hazard ranking of ENM with HT/CS3, in a study first of its kind. Furthermore, using novel high throughput imaging approaches and advanced image analysis software multiple biological endpoints can be investigated, and in some cases in real time, in cell cultures and in zebrafish embryos. The availability, via the European Zebrafish Resource Centre (EZRC)[4], of thousands of mutants and transgenic lines which have specific gene alterations facilitates enormously the identification and confirmation of toxicity pathways.

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.

The importance of the protein corona formed around nanoparticles upon contact with biological fluids or living organisms has recently been highlighted1, and it is now understood that it is not the bare nanoparticles that interact with living systems but rather the biological interface conferred by the adsorbed biomolecules that organisms actually "see", with the nanoparticle acting only as a scaffold [5, 6]. This corona is, when sufficiently long-lived, thought to govern the particles' biological fate. However, even this long-lived "hard" corona evolves and re-equilibrates as particles pass from one biological fluid to another, which may be an important feature for long-term fate. It has recently been shown that transfer of nanoparticles from one biological fluid (plasma) into another (cytosolic fluid), used as a simple illustrative model for the uptake of nanoparticles into cells, resulted in significant evolution of the corona in the second biological solution, but the final corona contained a "fingerprint" of its history [7] .

An important hypothesis is that this evolution could be used to map the transport pathways utilized by nanoparticles, and eventually to predict nanoparticle fate and behaviour based on characterisation of the initial corona in a representative biofluid. A similar concept for MNMs exposed via aquatic or terrestrial media containing natural organic matter (NOM, initial corona) taken up into organisms (final corona) has also been shown to exist [4, 5] and needs to be further investigated.

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 a biota in a nanoparticle specific manner where toxicity is one of the outcomes of these interactions. Others may include reduced energy reserves, reduced fitness and ultimately increased vulnerability.*

The current state of the art in this arena has advanced to the point where some patterns of toxicity emerge and there is understanding of internalisation of MNMs in biota. Recent advances also include novel tracers (stable isotope labelled MNMs) and better understanding of alternative sources of uptake (food versus water) by biota6 1. There is however currently no overarching framework for risk assessment.

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.

There are currently no dedicated toxicity tests for MNMs in the soil environment, and NanoMILE will develop a dedicated demonstration study, by an industry partner, adapting findings from this WP.

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.*

Currently there is extensive state of the art on MNM toxicity that is obtained by in vitro studies. Such *in vitro* studies are very useful for identification of toxic potency and mechanistic studies, and can support the outcome of *in vivo* studies. However, the information does not fit well in risk assessment.[8] In addition, the availability of *in vivo* repeated dose toxicity studies is limited.[9] Such *in vivo* data are therefore urgently needed, as are new paradigms based on low doses and closely linking toxicology and biokinetics.

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.

The objectives will be realised by using realistic inhalation, oral and intravenous exposure scenarios, mimicking occupational, dietary and medical use of MNMs. Specific attention will be paid to low dose exposure and long term effects and to what extent short-

term toxicity testing plus toxicokinetics can predict the outcomes of long term exposure. In particular the predictive value of this approach for tissue accumulation will be assessed. In addition to assessments on local adverse effects of MNMs at the port of entry via routine pathology and biomarker evaluation, analyses of systemic toxicity, including effects on the immune, cardiovascular and central nervous system will also be determined.

In vitro experiments will be focused on identification of mechanisms involved in the acute toxicity for various endpoints (cell death, cytokine induction, oxidative stress, DNA damage (repair), proliferation, DC maturation etc.) and using models for identification of migration of MNMs across cellular barriers, whereas *in vivo* experiments (acute and repeated dose up to 28 days) will focus both on local effects depending on the exposure route, and systemic effects, especially immunotoxicity, neurotoxicity and cardiovascular effects, including models for diseases (allergy to proteins and low-molecular weight chemicals, atherosclerosis, deficient biological barriers, neurodegenerative disease).

Toxicokinetic experiments will be performed to evaluate MNM translocation and migration as predicted from in vitro models. Different routes of exposure will be explored, as well as the particle-characteristics that determine translocation (e.g. particle size and charge, presence of (protein-) coatings). The results for selected parameters will be evaluated against results obtained in non-mammalian species (zebrafish or *C. elegans*, see above) as proxy to determine possibilities for applying one of the 3Rs for alternative testing of the safety of MNMs.

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 toxicants1. 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. Research by WP leaders [10, 11] has shown that these methodologies can be tremendously effective in biomedical research where they have already contributed to identifying networks predictive of clinical response, drug resistance and novel therapeutic targets. The dynamical models will also enable *in silico* simulations of the toxicity responses to MNMs, which will be tested experimentally.

WP8 will encompass several species/cell type spanning ecotoxicology and human toxicology, including algae (model plant), daphnia (model invertebrate), zebrafish (model vertebrate) and a human cell line. All this work is completely novel and represents advancement of the state of the art, both in scale and detail. At the same time the work is achievable being supported by other WPs (notably WP2 and WP4), and through the data integration and management capabilities of an industrial partner.

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.

Current understanding of MNM mode of action suggests there may be specific physicochemical features in MNM design that confer or influence toxicity. Such features or descriptors include aspect ratio ("asbestiform" MNMs or HARNs[1]), surface modifications and their stability, (oxidative) reactivity, hydrophilicity/hydrophobicity and size [12, 13]. More novel descriptors such as band gap have also been evoked.[14]

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

[1] High aspect ratio nanomaterials is a major focus for project NanoReg, and it has been decided to exclude from NanoMILE to avoid overlaps. However, high throughput work will cover HARNs and thus link up with NanoReg results.

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.

QSARs, perhaps more appropriately termed QPARs (as it is physicochemical "properties" rather than "structures" that need to be linked to a specific mode of hazardous activity) will form a fundamental component of NanoMILE. There are two main difficulties related to the development of nano-QSARs: The first is lack of sufficiently numerous and systematic experimental data and the second is the currently limited knowledge on mechanisms of toxic action. The former is being addressed in a number of major EU funded projects, data from which will feed directly into NanoMILE, via common project partners. The latter will be addressed within NanoMILE and knowledge acquired will transfer to this WP. An understanding of the relationship between the physical and chemical properties of the nanostructure and their *in vivo* behavior would provide a basis for assessing toxic response and more importantly could lead to predictive models for sub-classes and OECD recommends1 a procedure for the grouping of chemicals.

The overall objective of nano QPAR models is to relate a set of descriptors characterizing MNMs with their measured biological effects, for example, cell viability, or cellular uptake. Such models can then be applied to newly designed or commercially available MNMs in order to quickly and efficiently assess their potential biological effects.

The integration of technology and risk assessment with life cycle perspectives enables to identify innovation pathways for sustainable and responsible nanomaterials. With an integrated technology assessment NanoMILE aims to identify opportunities of new materials by integrating the results from all other work. We are linking the state of knowledge in research with the innovation processes in industries to facilitate sustainable innovation.

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 by 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.

The 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.

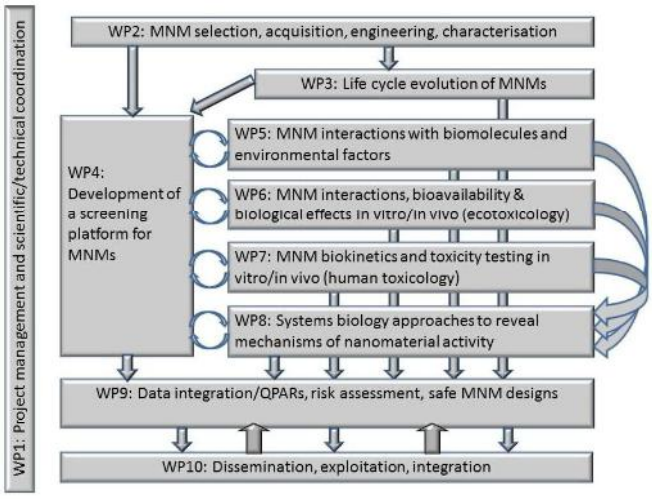

Figure 1. NanoMILE WP flow diagram and interdependencies

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, so as to address the call topic "NMP.2012.1.3-1: Systematic investigations of the mechanisms and effects of engineered nanomaterial interactions with living systems and/or the environment" in its entirety and at the highest technical level.

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.

5 Progress and Outcomes to date

All workpackages are progressing well, with a range of exciting outputs presented at the 1st Annual meeting held in Antalya, Turkey in conjunction with NanoTox2014. The first technical deliverables, including reports on the Phase 1 particle library, the approaches to ageing of nanomaterials, and the approachces for time-resolved characterization of nanomaterials have been delivered. Summaries of some of the key outcomes to date are reported below.

Selection of Nanomaterials

NanoMILE aims to develop mechanistic models for the interaction of MNMs with biological systems and in the light of this approach the selection of the materials for study will originate from a consideration of physico-chemical properties rather than the more commonly adopted approach of simple chemical compositions, industrial scale or perceived commercial relevance. The final selection of MNMs for mechanistic assessment of their interactions with living systems was based on a consideration of a number of factors, including:

1) The hypothesised mechanism of biological activity.
2) The availability of MNMs via one of the NanoMILE sources.
3) The feasibility of systematically modifying key physicochemical parameters for the basic material type.
4) The availability of existing toxicological data (avoiding duplication).
5) The identification of gaps in existing materials evaluation.
6) Special needs such as for MNM detection and labelling (stable or fluorescence).

To achieve this, the MNM libraries to be developed in the NanoMILE project have been selected following careful review of other relevant projects (see review of current literature included in Deliverable Report D2.1), commercial availability as well as expertise and capabilities available within the NanoMILE project. The NanoMILE project aims to be comprehensive in its coverage of MNMs, and thus a wide range of MNMs will be selected from the major classes of materials - metals, metal oxides, carbon based structures, functionalised structures and core-shell structures.

A tiered MNM procurement/production strategy is being adopted within NanoMILE, as follows:

- A first group of MNMs has been sourced from variety of external sources (industry, commercial, research repositories, MNM libraries from other EU funded programs/projects as well as national/international standards organizations) and will be rapidly available to the partners for establishment of test protocols and harmonisation of approaches within and across WPs.
- A second group of MNMs will be produced by appropriate chemical or physical modification of selected members of the first group of MNMs. Key partners in WP2 will be involved in these modifications
- Finally, a third group of specialised and highly tailored materials will be fully synthesised in-house by selected project partners. Here the focus will be on development of nanoparticle libraries and on systematically designing out key properties linked to toxicity (e.g. dissolution or positive surface charge) and assessment of the effect of such changes on other physico-chemical properties and on biological impact. For example, it is conceivable that changing the dissolution potential of an MNM may introduce other toxicity features such as biopersistence, and these aspects will also be assessed, and recommendations regarding optimal design criteria 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 testing such features by designing them in or out (both at bench and pilot scale);
- b) developing models of quantitative structure (property) –activity relationships (QS(P)ARs) enabling predictive work to evolve and feed into risk assessment; and
- c) providing an integrated platform for risk assessment.

All materials procured or developed within NanoMILE are being subjected to extensive physicochemical characterization using state-of-the-art methods (imaging, compositional and structural, and following where possible established (e.g. QualityNano, NanValid, NanoReTox) protocols, thus avoiding problems of unreliable cross referencing of experimental results.

By adopting this three-pronged strategy NanoMILE intends to produce a sufficiently large matrix of MNMs in a relatively short time period so as to be able to benefit adequately from the availability of the high throughput test facilities which are a key aspect of this project. The procurement and production activities will be conducted in parallel.

Table 1: Initial selection of MNMs for assessment in NanoMILE and key descriptors that will be systematically assessed / varied.

Nanomaterial	**Justification for selection**	**Key descriptors**	**1st group (commercial)**	**2nd group (modified)**	**3rd group (bespoke)**
CeO_2	Low solubility -> low toxicity Redox variations Isotopic label available Commercial value	Redox state Size Shape Solubility	Sigma JRC repository	Yes (PROM, UoB)	To be decided by Month 12 (MS3)
ZnO	High solubility -> high toxicity Isotopic label available High commercial importance (multiple applications)	Size Shape Dissolution rate / coating	JRC repository	Yes (UoB)	To be decided by Month 12 (MS3)
Ag	Variable solubility -> variable toxicity Isotopic label available High commercial value	Size Shape Dissolution rate / coating Surface defects	Sigma Sciventions Ltd.	Yes (JRC)	To be decided by Month 12 (MS3)
FexOy	Likely low solubility -> low toxicity Multiple structures & Magnetic properties Potential for labelling Medical applications	Crystal structure / phase Magnetic properties Coating Size	Sigma	Yes (PROM, N4I)	To be decided by Month 12 (MS3)
Graphene / other carbon-based MNM	High commercial relevance (e.g. Graphene Flagship) Non-spherical -> potential for alternative mechanisms of action	Aspect ratio Shape / structure C/O ratio / surface groups Surface functionalisation	Thomas Swan	Yes (CEA) (also negotiations with Graphene Flagship partners underway)	To be decided by Month 12 (MS3)

SiO_2	Easily fluorescently labelled Multiple synthesis routes Low toxicity generally, though evidence that structural transformations can induce toxicity (e.g. fumed silica)	Size Porosity	JRC repository IRMM standards BAM - Federal Institute for Materials Research and Testing	Yes (JRC)	To be decided by Month 12 (MS3)
TiO_2	Low solubility -> low toxicity Multiple coatings available Different crystal phases Commercial value Photoreactive	Crystal structure / phase Coating (ageing) Size ROS production	JRC repository NIST standards	Yes (PROM)	To be decided by Month 12 (MS3)

Towards a strategy for grouping and classification of Nanomaterials

A novel approach to identify interlinked physicochemical descriptors, and on this basis identify overarching descriptors (axes or principle components) which can be used to correlate with toxicity is proposed. 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 corrected 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 with 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.

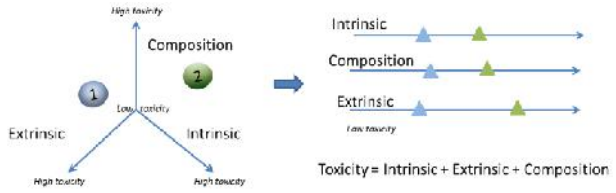

Figure 2. A principle components approach to predicting nanomaterial toxicity, where the experimentally determined physico-chemical parameters are mapped onto 3 axes (principle components), proposed here as and composition, intrinsic properties (i.e. those inherent to the nanostructure that are independent of the surroundings), and extrinsic properties (i.e. those 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), http://dx.doi.org/10.1016/j.nantod.2014.05.001.

Linking characterisation data to toxicity and ecotoxicity assays

A key feature of the NanoMILE project is the development of integrated datasets regarding mechanisms of NMs toxicity across a range of environmental species and cell models, and thus it is vital that the characterisation work undertaken here and in WP2 is closely integrated with the assays that will be performed in the other workpackages (WPs), including utilising

agreed media and serum (where appropriate). To this end, WP5 worked closely with WP4 and WP6 – WP8 to ensure that the selection of representative media for WPs 6-8 (MS10) was fed into the characterisation studies of WP5 related to characterisation of interactions of MNMs with biomolecules and environmental factors e.g. natural organic matter.

Based on this WP5 undertook time resolved characterisation of MNMs in different media – deionised water, PBS, 10% FBS dispersed in PBS, and Zebrafish embryo media (ZEM). These media were selected to provide a range of representative dispersions and to increase in complexity of composition and thus potential for impact on MNM dispersion quality and stability. Deionised water as a control, FBS (NanoMile centralized serum) used to represent conditions in biological systems, PBS as a standard buffer and zebrafish embryo media (recipe supplied by WP6) representing ecotoxicological media. Subsequent studies in WP5 will also utilise these representative media to ensure cross-comparability of datasets and facilitate data integration.

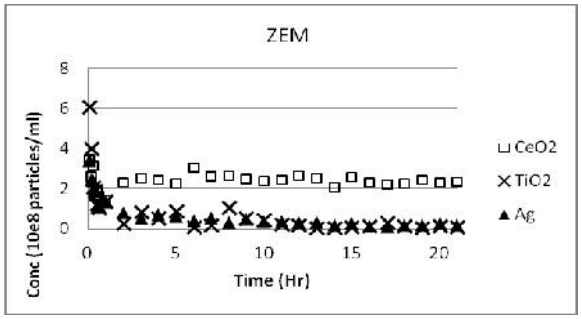

Figure 3. Graphs displaying concentration values for CeO_2, TiO_2 and Ag MNMs dispersed in Zebrafish embryo medium (ZEM) over a 21 hr duration.

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." [15]

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.[16] 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.

From the technical challenges identified above, and the workpackage structure designed to address these challenges, the NanoMILE consortium have identified a number of key outputs that will have significant impact for the various stakeholders involved in the nanosafety and nanocommercialisation question. Table 1 below summarises the key stakeholders for the outputs from the NanoMILE project, with whom targeted dissemination activities will be undertaken. An outline of the sorts of dissemination activities planned to address the needs of each stakeholder group is also given in Table 1.

6 References

1. http://www.oecd.org/officialdocuments /displaydocument pdf/?cote=env/jm/mono(2012)8&doclanguage=en

2. Gottschalk, F.; Nowack, B. (2011) Release of engineered nanomaterials to the environment. J. Environ. Monitoring. 13: 1145-1155.

3 Geranio, L., Heuberger, M., & Nowack, B., 2009. The behavior of silver nanotextiles during washing. Environ. Sci. & Technol., 43, 8113-8.

4. http://www.itg.kit.edu/ezrc

5. Walczyk, D., Baldelli-Bombelli, F., Campbell, A., Lynch, I., Dawson, K.A., What the Cell "Sees" in Bionanoscience. JACS 2010, 132, 5761-5768.

6. Lynch, I., Salvati, A., Dawson, K.A., 2009, Protein-nanoparticle interactions: What does the cell see? Nature Nanotechnol. 4, 546-547.

7. Lundqvist M, Stigler J, Cedervall T, Berggård T, Flanagan MB, Lynch I, Elia G, Dawson K. The evolution of the protein corona around nanoparticles: a test study. ACS Nano. 2011, 5, 7503-7509.

8. Park MVDZ, Lankveld DPK, Van Loveren H, De Jong WH. The status of in vitro toxicity studies in the risk assessment of nanomaterials. Nanomedicine 4, 669-685, 2009.

9. Priestly BG. Review of 2007-09 literature on toxicological and health effects relating to six nanomaterials. Scientific review report Australian Government, Department of health and Aging, NICNAS 2011.
http://www.nicnas.gov.au/Current_Issues/Nanotechnology /Mon_Lit_Review_of_NMs_of_Interest_PDF.pdf

10. Hines et al, 2010. Discovery of Metabolic Signatures for Predicting Whole Organism Toxicology TOXICOL. SCI. 115, 369-378.

11 Williams et al, 2011. Towards a System Level Understanding of Non-Model Organisms Sampled from the Environment: A Network Biology Approach. PLOS COMPUTATIONAL BIOLOGY, 7, e1002126.

12. Auffan et al., 2009, Towards a definition of inorganic nanoparticles from an environmental, health and safety perspective, Nature Nano, 4: 634-641

13. Nel et al, 2006, Toxic potential of materials at the nanolevel; Science, 311: 622-627.

14. Zhang, H. et al (2012) Use of metal oxide nanoparticle band gap to develop a predictive paradigm for oxidative stress and acute pulmonary inflammation. ACS Nano on line, 10.1021/nn3010087.

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