NanoMILE

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

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5	Joint Research Centre of the European Commission	JRC	Belgium
6	Eidgenössische Materialprüfungs- und Forschungsanstalt	EMPA	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 parent MNMs or to MNMs that have been incorporated into products and subsequently released, either in their original or altered form by 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 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.

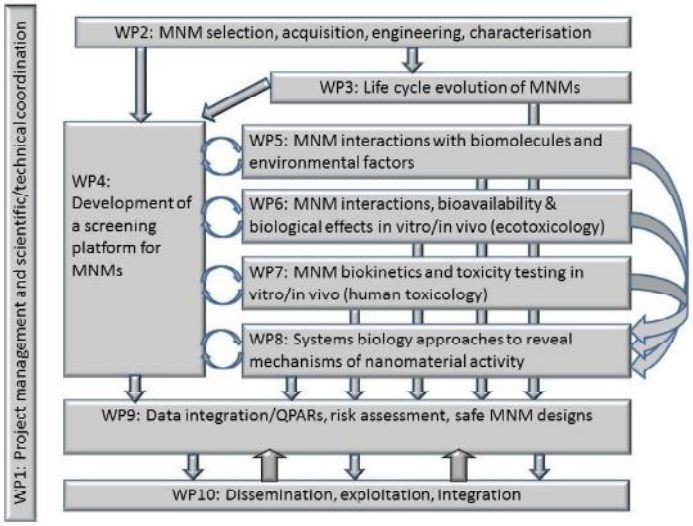

Figure 1. NanoMILE WP flow diagram & interdependencies

5 Progress and Outcomes to date

The middle period of any large project is where the bulk of the activity happens, and NanoMILE is no exception: all 8 technical WPs have been fully operational throughout the period, and all 28 partners have been actively engaged. All 6-monthly face to face meetings, in Copenhagen (M18), Brussels (M24), Athens (M30) and Edinburgh (M36) have been exceptionally well attended with over 60 NanoMILE researchers, facilitating internal WP discussions and planning, as well as cross-WP integration and alignment, and designing the integrated NanoMILE grouping and classification framework that forms the final project outcome.

The 10 industry partners have been fully integrated into the NanoMILE research activities, contributing actively to WP2 (PROM, N4I; particle synthesis / manufacturing), WP5 (Malvern, Attana; method development for characterization of particle-biomolecule and particle-cell interactions), WP6 (Eurofins, ecotoxicity of pristine and aged NMs), WP7 (BASF, Vitrocell; inhalation exposure, including a new Air-liquid Exposure device); WP8/WP9 (Biomax, Novamechanics; data management and QSAR development) and WP10 (EU-vRI; dissemination and exploitation). A dedicated section of the NanoMILE website is

industry-facing and devoted to showcasing NanoMILE's products and services with and for industry.

In the previous edition of the Compendium we featured some of the key early publications from the project rather than giving an update on each WP. This approach was adopted because the different WPs they were at different stages of development. However, with 27 publications in the last year or so, it was not possible to summarise them in just a few pages, so we provide a brief update on the highlights from each WP. The full list of the NanoMILE publications to date (39 and counting) is available via the NanoMILE website.

All workpackages have progressed well with a range of exciting outputs and scientific discoveries. Highlights are provided below.

WP2 - MNM selection, acquisition, engineering, characterization has played a central role in the succees of the project to date. With regard to the work in Task 2.3 and Task 2.5 during the current reporting period all WP2 partners have continued to be active in contributing to the preparation of new or re-supply of nanomaterials for use in WP 3-8. In line with the planning in the DOW the contribution of all partners to the materials supply tasks has progressively decreased in the period up till M36. The majority of the materials supply work has now been completed. No new materials are planned for production in the next period although when necessary and agreed with the relevant partners re-supply of materials may be undertaken. With respect to the Task 2.4 "Characterisation of MNMs" all particles produced by UoB, as well as those received from industry partners have been subjected to basic physico-chemical characterisation by UoB to determine size, shape, zeta-potential and crystal structure. Where specifically requested by WP4-8 partners, tests of the stability of the different particles in the various media utilised in WPs 4-8 have been addressed. In the cases of materials prepared by JRC and CEA, basic characterisation was undertaken using their own in-house facilities. At this time the experimental studies for Task 2.4 have been largely completed and the results are being integrated into a single reference document for use by the consortium. This document which constitutes the Deliverable 2.5 "Characterization files for all 3 libraries of MNMs" currently contains over 650 pages of data characterizing the NanoMILE MNMs libraries.

WP3 - Life cycle evolution of MNMs was also in part a service WP, providing "aged" MNMs to other WPs. WP3 focused on ageing of MNMs in air, water and products, incuding sulfidation, phosphidation, role of fulvic acid (FA) and impact of UV. All WP3 tasks are completed. Highlights include the success of the ageing protocol developed for Ag-MNMs (EMPA) to achieve complete sulfidation of Ag-MNM, but the chemical composition was not the only parameter to be altered during this transformation process, as the MNMs were observed to agglomerate significantly, to form a spider-web structure after aging. Comparing / contrasting the effects of pristine to altered MNM produced under these conditions, it may not be the chemical transformations alone which are responsible for different biological or ecological effects but also the physical structure may play a role. Transformation of MNM with natural organic matter (UoGen), which confirms that a FA coating acquired around CeO_2 MNMs is stable and irreversible, and is one of the key factors that will control, even in changing diluting conditions, transformation and toxicity of MNMs in aquatic systems (see figure 2). Moreover, when coated CeO_2 MNMs are present even in systems with increasing ionic strength (e.g. passing from fresh to coastal or marine waters) they resist aggregation in particular when monovalent salt is considered.

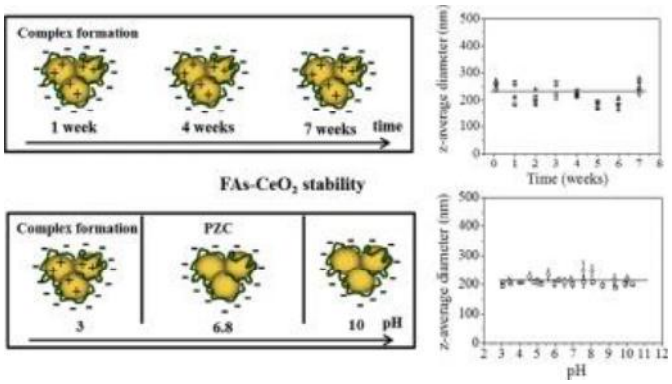

Figure 2: Effects of pH and fulvic acids concentration on the stability of fulvic acids – CeO2 MNMs complexes. From: Chemosphere, 2016, 114, 131–137.

WP4 - Development of a screening platform for MNMs has carried out systematic toxicity screening of up to 100 NanoMILE Phase 1 and 2 MNMs (with phase 3 MNMs ongoing currently) in a range of cell lines and zebrafish embryos against a range of endpoints to identify lead candidates for WP 6-8, and for interesting candidate MNMs is further developing novel, robust toxicity assays for HT/CS of MNMs as well as identifying common biomarker profiles (from WP 6-8) across multiple species. The first list of MNMs was selected on the basis of having similar size but different chemistry and coatings, while the second screening panel included the aged MNMs (from WP3), the series of Zr-doped CeO_2 MNMs developed for WP7, and a series of 41 ultrasmall particles (diameter ≤ 20nm). The MNMs in the first list were tested in different mammalian cell lines and zebrafish embryos to link physico-chemical properties to multiple adverse effects in different biological systems. The cell lines represent different organs (liver, lung, colon, immune system). Dispersion and dilution of the MNMs was done according to an agreed standard operation procedure, and the doses tested ranged from 1-125 µg/ml which corresponds to cell surface area doses of 0.3-9.1 µg/cm². Pure medium was used as negative control and amine-modified polystyrene ($PS\text{-}NH_2$) NPs as positive controls. The assays used are based on high-throughput/-content (HT/C) techniques. End points such as cell count, cell membrane permeability, apoptotic cell death, mitochondrial membrane potential, lysosomal acidification and steatosis have been studied in cells. The zebrafish embryos were tested for hatching rate, malformations and mortality. Integrated multi-partner publications are in preparation at present.

WP5 - MNM interactions with biomolecules and environmental factors is tasked with furthering our understanding of what is presented on the surface of the NanoMILE MNMs when dispersed in relevant biological media (e.g. the 10 % foetal calf serum used for the cell culture experiments and in vivo model fluids) in order to bridge from physico-chemical properties to

biological effects. A significant focus has been on developing automated and higher throughout approaches to assess MNM-biomolecule interactions. One example is the development of automated handling of sample preparation and Fluorescence Correlation Spectroscopy (FCS) measurement and data analysis, whose workflow combines a Labcyte Echo Liquid handler with an automated FCS Setup and semi-automated data analysis. The setup allows preparation and readout of a 384 well plate in about one day with a 10 fold sample volume reduction (20µl per well). Measurement of a series of binding isotherms in a multi-component system is therefore possible. Preliminary experiments with TiO_2 uncoated MNM and Bovine Serum Albumin (BSA, Alexa Fluor 488) were performed and optimized, suggesting that BSA interactions with TiO_2 are not strong resulting in BSA having short residence times on the MNM surface. Other efforts include development of a surfactant titration method (see Figure 3) to study corona-MNM complex stability and allow selective separation of certain proteins from the corona.

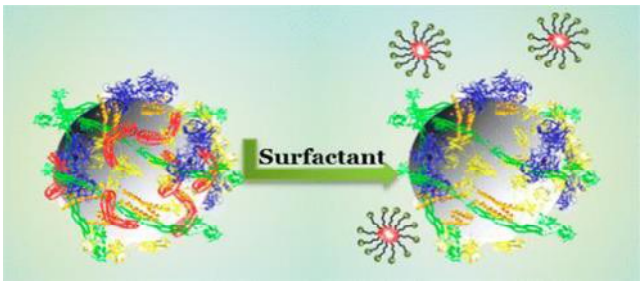

Figure 3: Surfactant titration to assess MNM corona stability. Anal. Chem., 2014, 86, 12055.

WP6 - MNM bioavailability & biological effects in vitro/in vivo (ecotoxicology) has tested a range of sentinel organisms to investigate the relative toxicity and organisms sensitivity to selected nanoparticles (NPs) that are currently suspected to have biological effects (e.g. nano silver). The test organisms included a freshwater algae (Clamydomonas reinhardtii), a freshwater fish (Danio rerio), and a range of terrestrial invertebrates including Caenorhabditis elegans, earthworms (Eisenia fetida), springtail (Folsomia candida), soil mite (Hypoaspis aculeifer), and the isopod (Porcellio scaber). Some tests followed standardised OECD test guidelines but new protocols better suited for studies on MNMs for those specific test organisms were also developed. Effects analyses were focused on apical endpoints from mortality, to development, growth, reproduction, neurological function (e.g. behaviour assays) and photosynthetic yield (algae). Of the MNMs studied only AgNPs were found to show toxicity at concentrations modelled for surface waters and surface soils in Europe, across the wide range of study organisms tested. Our results, together with the existing literature indicate that the release of Ag^+ and its uptake into the cell is the main cause of AgNP toxicity. For ZnO MNM dissoluton in the aquatic exposure medium was rapid and the predominant mode of toxicity of ZnO MNM is likely to be due to Zn^{2+} ions. However, no adverse effects were found for environmentally relevant concentrations of ZnO MNM in the terrestrial and aquatic organisms tested. No effects of CeO_2 or for TiO_2 were found on apical endpoints for any dosing level tested in the animals studied.

WP7 - MNM biokinetics and toxicity testing in vitro/in vivo (toxicology) is investigating selected MNMs to identify molecular mechanisms and pathways of toxicity. One hypothesis used in these studies is that the redox potential of the MNMs governs the toxicity. CeO_2 was chosen due to the fact that it can cycle between two redox states, Ce^{3+} and Ce^{4+}, which endows this MNM with catalytic properties, and suggests a mechanism of activity based on oxidative stress. The use of CeO_2 MNMs in vehicle catalysts makes it relevant for exposure via inhalation. Doping with Zr was used to alter the redox activity by incorporating ZrO in the crystal structure of the CeO_2 MNM (prepared by PROM, as per Table 1). The differences in crystal structure and redox potential did not result in large differences in to xicity (in in vitro and in vivo studies, papers in preparation) as the toxicity of the original CeO_2 MNM was relatively low. For the second phase, Fe_2O_3 MNMs doped with different amounts of cobalt. In vitro and in vivo studies are underway.

WP8 - Systems biology approaches to reveal mechanisms of MNM activity comprises the collection and deep analysis of "omics" Big Data associated with the biological responses of four model systems (Daphnia, Chlamydomonas, zebra fish embryos and A549 human cell line) to selected MNMs. Following earlier delays in the project related to the selection of MNMs and provision of aged MNMs for the deeper mechanistic investigation in WP8, progress in the wet laboratory exposures and omics data generation has been good. An array of omics responses have been measured, including gene expression profiling (using RNA Seq), metabolic changes (using mass spectrometry metabolomics) and lipid changes (using mass spectrometry lipidomics), as well as traditional phenotypic (apical) endpoints, for the four model systems. Data analysis is underway and further details will be provided in the report on the final period of activity.

WP9 - Data integration/QPARs, risk assessment, safe MNM designs has focussed on the development of a robust and predictive model to quantitatively define the correlation of the cell association of a set of gold NPs with their physicochemical properties and available data on protein corona fingerprints. We have computationally explored a data set that consists of 105 chemically diverse gold NPs with different surface modifiers and three different core sizes, namely 15, 30 and 60 nm. In the formulations 67 organic surface modifiers were used, including small molecules, polymers, peptides, surfactants and lipids that can be characterized as "neutral", "anionic" and "cationic" based on their chemical structure and net charge at physiological pH (pH 7.4). KNIME (Konstanz Information Miner) platform, a freely available and open source tool that is increasingly used for solving chemoinformatics problems (www.knime.com), was used to implement all steps required for model development and validation.

Code	Chemical composition	CeO_2:ZrO_2 ratio
CeO_2 A	CeO_2	100: 0
CeO_2 B	CeO_2 + ZrO_2	86: 14
CeO_2 C	CeO_2 + ZrO_2	73: 27
CeO_2 D	CeO_2 + ZrO_2	48: 52
CeO_2 E	CeO_2 + ZrO_2	22: 78
CeO_2 F	CeO_2 + ZrO_2	8: 92
CeO_2 G	ZrO_2	0: 100

Table 1: Series of Zr-doped CeO_2 MNMs.

WP10 - Dissemination activities

NanoMILE partners have been active in terms of disseminating their activities within NanoMILE from the outset of the project, with period 2 being especially active: The table shows the period 2 and total dissemination activities, including 39 papers and 107 conference presentations. NanoMILE partner chair several NanoSafety cluster (NSC) working groups (WGs) including Hazard (WG2), Standardisaiton Sub-group (WG7) and the newly formed Safety-by-design (WG9), as well as representing WG7 Dissemination on the NSC Steering Committee.

Dissemination Activity	Period 2	Total
Conference talks/posters	76	107
NSC activities*	6	12
Journal articles	27	39
Standardisation activiteis	0	3

* Chairing WGs, organising events/compendium, presenting @ events, Newsletter articles, etc.

NanoMILE Cooperation with other projects/programmes

NanoSolutions (FP7)

NanoSolutions is our sister project, addressing the same call topic as NanoMILE, meaning there are losts of potential synergies. A joint meeting was organised at the UoB office in Brussels in July 2015, and work is underway between the two projects to draft a joint paper on the selecton procecess the projects unferwent to determine which NMs, which model systesms and which methods/assays to utilise, which will form an important part of the scientific record at the present time in nanosafety.

Additionally, the two projects are co-arganising their final meetings (together with GuideNano and SUN) in February 2017 in Malaga. This will provide an important opportnty for data and knowledge sharing and a consensus report will be drafted from the meeting.

NanoFASE (Horizon 2020)

Given NanoMILE's focus on hazard and NanoFASE's focus on exposure and fate, there is a natural complementarity between the projects, which is being harnessed through common MNMs where possible (e.g. from Prometian particles, a partner in both projects), and through the extension of the NanoMILE KnowledgeBase to cover NanoFASE particles and datasets in additioan to NanoMILE's datasets. This will support integration across data for complete risk assessment in due course. NanoMILE and NanoFASE are organsing a joint workshop / Training School for Autumn 2016.

SHYMAN (FP7)

There have been crossovers between NanoMILE and another FP7 project which PROM is involved in - SHYMAN (Sustainable Hydrothermal Manufacture of Nanomaterials, Grant Agreement Number 280983). As part of this project, selected materials which PROM currently manufactures will be destined for scale up to be produced at industrial quantities – up to 1000 tonnes per annum. It is in the interest of the NanoMILE project to study nanomaterials which are industrially relevant, while it is in the interest of the SHYMAN project to consider the toxicological impact of these engineered MNMs on living systems and the environment (especially when considering accidental release or disposal during the production phase). Therefore, PROM has been acting to feed information and samples between the two projects which has resulted in toxicology data, obtained by the JRC within NanoMILE, being used in a Deliverable Report within SHYMAN (Deliverable 6.4: Identify potentially high environmental and economic impacts of the hydrothermal synthesis process to be submitted by Czech Technical University). The JRC and the NanoMILE project have been fully credited for their work.

eNanoMapper (FP7)

NanoMILE is working with eNanoMapper in temrs of developing the ontology for MNMs ageing, MNMs coatings etc., as well as supporting the development of the database and its sustainability. NanoMILE gave a stimulus presentiaton at the eNanoMapper databases workshop in Brussles in January 2016, and hosted a joint meeting with eNanoMapper team members as part of the M36 meeting in Edinburgh. UoB and eNanoMapper partner have jointly developed a starting communities bid for a research infrastructure (submitted April 2016) as well as for an e-infrastructure (1 stage proposal, submiited April 2016).

NanoDefine (FP7)

NanoMILE is actively contributing to NanoDEefine's project networking activities, including presenting the project's activities and potential contributions to community activities at the NanoDefine meeting in February 2016. NanoMILE completed the templates and other reporting requested, and will continue to enagage as new initiatives emerge.

Nanosafety Cluster

NanoMILE are playing a leadership role in the NanoSafety Cluster (NSC), as one of the larger projects running at present. To this end, we continue to contribute to NSC activities, such as editing the NSC compendium for 2014 (Iseult Lynch), the contributing to the organisation of the 2nd NSC young-researchers meeting in Visby (Profs. Valsami-Jones and Lynch are on the organising committee), as well as providing leadership of specific WGs: Flemming Cassee leading WP2, Benoit Hazebrouck leading WG7 sub-gorup on Standardiation, Eva Valsami-Jones leading the new Safety-by-Design Wg, and Isuelt Lynch representing Dissemination on the NSC Steering Committee.

EU-US Communities of Research

NanoMILE have also been active in the EU-US CORs, with two NanoMILE parnters presenting at the 2013 CORs workshop in the US (Denise Mitrano (EMPA) and Francesco Falcini (University of Birmingham / University of Liverpool). NanoMILE partners are actively involved in the regulatory, databases &

curation and exposure CORs, and will be involved in the methods and characteriation one in due course.

Nanotechnology Data Curation Initiative

NanoMILE have signed-up to act as a Stakeholder Liaison for the EU-US CoR Database & Curation / NCIP Nanotechnology Working Group "Nanotechnology Data Curation Initiative". As part of this, NanoMILE will provide input and responses to six themed questionnaires over the next 18 months, which will (along with the inputs from the other liaisons) collectively form the "landscape" of nanomaterial data curation. This also helps to ensure that NanoMILE has international visibility and that the approacjes being pioneered within the NanoMILE knowledge base are linked to international efforts in this arena. The first co-authored paper has just been accepted in NanoScale.

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 further crystalized our next steps towards exploitation and our Standardisation planning is advancing in parallel. Based on our selection criteria (existence of a need, including for industry and regulators; technical readiness; organisational readiness) 3 work items were selected, namely: Stable-isotope labelling of MNMs, Isopods as a model for bioaccumulation of MNMs and Toxicokinetics of MNMs.

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7 Directory

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