Executive Summary for NanoInteract Second Annual Report

NanoInteract: Development of a platform and toolkit for understanding interactions between nanoparticles and the living world

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The responsible development and implementation of nanotechnology is recognized as a common desire across the European arena of research, and far beyond. The overarching objective of NanoInteract is to create a firm scientific and technical basis for understanding and potentially prediction of likely biological impacts of engineered nanoscale particulates. NanoInteract is an EU FP6 STREP funded under the NMP theme, running from January 1st 2007 until 31st December 2009. The project partners are listed below, and the website is www.nanointeract.net.

To date the program has lead to no newly identified hazard (solely due to nanoscale elements) for nanoparticles, but it has highlighted several issues requiring further investigation, in particular related to the need for consideration of appropriateness of several of the established OECD (and other) tests for chemical toxicity to assessment of nanoparticle toxicity, and the myriad ways in which the presence of nanoparticles and nanoparticle aggregates can impact on the tests and their interpretation. The knowledge and experience gained is now forming the basis for other projects, both in the EU and across the world.

Partner	**Country**	**Status**
National University of Ireland / University College Dublin	Ireland	University
Ludwig-Maximilian Universität	Germany	University
Oxford University	UK	University
Trinity College Dublin	Ireland	University
University of Ulster	UK	University
Université Paris-Sud	France	University
Lund University	Sweden	University
National Institute for Public Health and the Environment	Netherlands	Research Centre
Nofer Institute of Occupational Medicine	Poland	Research Centre
Ghent University	Belgium	University
Rice University	United States	University
Glantreo	Ireland	Industry
Medtronic	Netherlands	Industry
L'Oreal	France	Industry
Intel	Ireland	Industry
Umicore	Belgium	Industry
DSM	Netherlands	Industry
Weizmann Institute	Israel*	Research Centre

The procedure to add Weizmann Institute as a partner was begun in Period 2.

The NanoInteract programme acknowledges that the field of nanosafety assessment is a new field, with no established paradigms or standard practices or protocols, and recognises its responsibility to ensure that any contributions to the literature that emerge from the NanoInteract project must be of the highest scientific quality, and must be internally checked and confirmed, so as not to add further confusion to an already slightly complex arena. The depth and breadth of the issues are unique, and the foundation of the field on a rational and quantitative basis is of paramount importance for all concerned, from governments to NGOs to industry and beyond. As there are still so many unknowns, a key aspect of the NanoInteract programme is the use of internal round robin type approaches, whereby important results are reproduced and replicated in at least one completely separate laboratory using the identical protocols and starting materials, in order to ensure that the results are scientifically valid and reproducible. In addition to addressing known risks from nanomaterials, such those endpoints for which there are established mechanisms of action (e.g. DNA damage), NanoInteract is also addressing the potential for new toxicological end-points, such as nanoparticle-induced changes in protein aggregation and fibrillation, as well as changes in subtle gene and protein regulation pathways.

In addition to the overarching issues outlined above, there are also significant problems of a more technical nature relating to the use and handling of nanomaterials which cast uncertainty on current methods and approaches. Important objectives of the NanoInteract programme are therefore to produce controlled and reproducible dispersions of nanoparticles in biological fluids, reproducible presentation of nanoparticles to cells, and to understand these and other issues related to nanoparticle dose and response. In particular the program seeks to indentify and control all of the issues specific to the physicochemical and nanoscale nature of nanoparticles in biological solution that could lead to false results or mis-interpretation of results from toxicity tests, and to develop protocols that account for, address or eliminate such complications.

Work Program

NanoInteract seeks to connect the uptake and bio-distribution of nanoparticles by cells with the nature of the particles in biological solution via the biomolecule corona, and to the functional impacts f the presence of the nanoparticles in specific sub-cellular compartments. Within the programme, a variety of trophic levels and organisms complexities are considered, ranging from cells, algae, daphnia magna, and others. This knowledge is then connected to the outputs from classical toxicology testing (such as those relevant to REACH and OECD considerations), in order to evaluate whether these existing toxicity tests are sufficient to predict nanoparticle toxicity, or whether they need to re-designed, or supplemented with newer approaches more tailored to the issues implicit in the nanoscale. Fundamental to the project is the effort to establish protocols and standards via which every step of the project are being controlled as we seek to eliminate the factors that currently causing irreproducibility in dose and subsequent response to exposure to nanoparticles. An overview document of these protocols will be published as an output of the project to enhance progress in the field and to share our experiences in this arena with others.

The NanoInteract objectives and challenges can be summarized as follows:

- To establish experimental protocols for every aspect of the study of nanoparticle interaction with cells, and several types of aquatic plants and organisms, ensuring complete reproducibility.
- To understand effect of adsorbed protein on nanoparticle stability and nanoparticles on protein conformation and function, ultimately connecting this to biological impacts.
- To connect cellular location of nanoparticles with intra- and inter-cellular processes disrupted.
- To combine these results, along with the expertise from diverse disciplines, to point towards a ‘standard approach to nanotoxicology’.

Work performed during period 2 and some highlights of the outcomes

The NanoInteract project has progressed well during the second year of activity, with increasing focus on scientific excellence and the generation of scientific data addressed towards the key knowledge gaps in the area of nanosafety and understanding the mechanisms of nanoparticle interactions with living systems. However, one should not be complacent. The challenges in this project were much greater than foreseen, and the level of discipline and focus on quality and standards still needs to be further developed.

A key aspect in the success of the NanoInteract project to date has been the development of secure processes for controlling the systems used across all partners. In year 2 of the NanoInteract project, the investigations have focussed on several key classes of nanoparticles – silicon dioxide, cerium oxide, aluminium oxide (both particulate and monolithic surfaces), polystyrene, quantum dots and gold nanoparticles. Not all studies have been performed on all particle types, but certain particles have been carried through the majority of the studies in order to have a complete story. In this case, the test particle is the silica dioxide, which also has significant commercial interest, as it is already in use in industry.

Achievements of significant practical importance from the second year of the project include:

- Establishment a solid common ground and understanding for comparing the results from the very different groups and experimental approaches used in the NanoInteract project. This is quite a significant challenge in many cases, as each cell line or test often requires a different growth medium with different salts and pH, all of which affect the stability of the nanoparticle dispersion, and as such complete characterisation of the nanoparticle dispersions in each medium is necessary. This is a significant draw-back of the ecotoxicology testing framework, as well as restricting direct comparison between different cell lines. Within the NanoInteract programme, we have overcome this limitation by having (where possible) a single protocol, and where this is not possible to compare the recommended protocol to our protocol. While this significantly increases the effort required, it also significantly increases the reliability and reproducibility of the outcomes and results.
- First cross-institutional example of a round-robin type toxicology experiment, where identical results were obtained in two separate institutions using an identical protocol, and common cells, serum and nanoparticles. The test used was the COMET assay for genotoxicity, and the particles used were silica nanoparticles from a commercial source (Sigma) and from within the NanoInteract consortium (Glantreo), in a range of nominal sizes (10-400nm) and with different surface properties (surface charge and coating). Using the 3T3 cell line, no genotoxicity was observed and the same answers were obtained in all laboratories. One year of intra-laboratory visits was required before the problems with the round robin were identified.

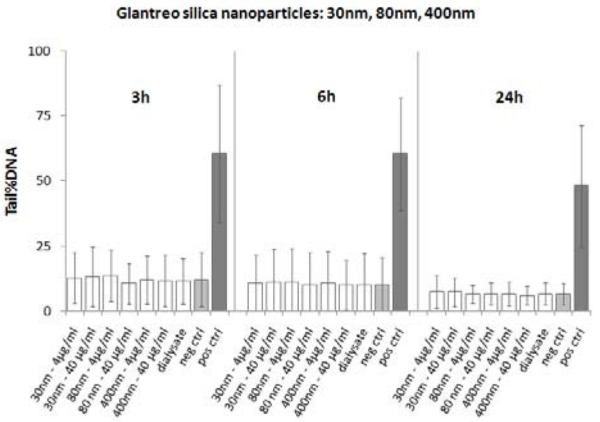

Figure 1 Comet assay of Glantreo silica nanoparticles. Note that the 6 hour time-points are the mean of the results from the studies at the two independent locations.

- The huge variability between batches of nominally identical particles from the same source was not fully appreciated when first writing the project description, but we have since learned that this is not an insignificant issue in determining nanosafety. For example, we have found that different batches of positively charged polystyrene from the same supplier and with identical specifications induce different degrees of cellular apoptosis in identical cells at identical nanoparticle doses (50 µg/mL). The implications are profound for the field in general.
- We now have growing quite serious concerns that the quality and standards of common nanomaterials are insufficient to be sure that we are seeing true nano-impacts. We have evolved a set of processes (not fully satisfactory yet) to clean and validate commercial samples.
- The concept of the biomolecule corona, which was first outlined in the NanoInteract project, has gained considerable interest and support in the international scientific community, and was

awarded the US National Academy of Sciences Cozzarelli Prize in 2008. After considerable challenges (again in reproducibility) a standard technique and protocol has been developed to deduce the major components of the nanoparticle-corona. We are discussing how this could be developed as a standard characterisation in conjunction with IRRM and NIST for their silica and gold standard (reference) nanoparticles. NanoInteract is also developing methods to quantify the nanoparticle protein corona, and following nanoparticle uptake by cells.

- Significant advances are being made towards understanding the functional impacts of nanoparticles in terms of elucidating the signalling pathways utilised by cells during the uptake processes, and the toxicity pathways induced, such as the route of apoptosis induced by positively charged polystyrene nanoparticles. These are now being correlated with effects on upstream protein signalling, mid-stream gene expression, and downstream protein expression.
- There is a small but clear warning of the need to reconsider simplistic assertions about nanoparticle aggregation/agglomeration in the environment. Natural organic matter in river water successfully de-agglomerates nanomaterials we have been unable to disperse in the lab. We have ideas why, but that is future science.
- The first quantitative results of nanoparticle uptake have been produced, and (to our knowledge) the fist computational model of uptake fitted to the experimental results. We are a long way from full modelling approaches, but the conceptual step has been made. Processes such as uptake have been codified by "rate constants" from a model fit, and we are entering a period like that in which classical chemistry was reduced to reaction kinetic constants.

Overall, the experience of NanoInteract leads us to emphasize the challenging nature of the problems in assessing the potential risks posed by nanoparticles for living systems, which require highly focussed and large multidisciplinary teams that can address all the issues. Significant results are emerging, but it is now clear that to carry out truly useful science in this field, one will need to ensure that a number of different laboratories obtain precisely the same results. This transpires to be a major (but achievable) challenge, but without it, one has to consider carefully the value of publishing the results, and the implications for others seeking to reproduce results.

It is hard to predict all the specific research outcomes of NanoInteract, but it is clear that a major element will be an understanding of *how* to carry out durable, reproducible, collaborative research in the field of nanotoxicology. It also seems likely that the nature, and to some degree the role of the 'protein corona' will clarify and may become a standard characterisation step for determining nanoparticle impacts.

Teaching and Training; A significant element of the program, not fully foreseen in the original plan, has been the consequence of quite different background and training of different students and researchers on the ground in the laboratories. Communicating a common understanding of the need for protocols, the variability of samples and the need for controls at all levels has been a significant issue. A training workshop on nanoparticle dispersion was held in conjunction with the Annual Meeting in Lodz in March 2008. The current informal protocol documentation developed across the entire programme will be formalised and transmuted into a training handbook and an associated training course, during activity period 3.

Dissemination; Results are being disseminated as appropriate in the literature, with emphasis on high impact journals and papers from several partner organisations. Additional dissemination routes involve conferences, working group in EU, OECD, and other instruments. Significant advances from NanoInteract were communicated via the NanoImpactNet conference in Dublin, the NanoSafe conference in Grenoble, the NanoRisk meeting in Paris. A key output in Activity Period 2 has been the establishment of the International Alliance for NanoEHS Harmonisation (IANH, www.nanoehs-alliance.org). At the end of the second activity period, 22 articles have been published from the NanoInteract consortium, and an additional 10 manuscripts are submitted or in the final stages of preparation.