Executive Summary for NanoInteract Third 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 being key to recouping the significant investment in Europe into nanoscience and nanotechnologies over the last decade. Nanotechnologies are considered an enabling technology, as their potential applications are so widespread, and indeed several hundred consumer products claiming to contain nanotechnologies are already available. Central to achieving this vision is ensuring that nanotechnologies do not cause inadvertent harm to human or environmental health at any stage of their life cycle.

Thus, the overarching objective of NanoInteract is to create a firm scientific and technical basis for understanding and potential prediction of likely biological impacts of engineered nanoscale particulates. NanoInteract was an EU FP6 STREP funded under the NMP theme, running from January 1st 2007 until 31st December 2009, and as such is now completed with some quite significant success. The project partners are listed below, and the website is www.nanointeract.net.

The focus in period 3 of the project was on deepening our understanding of the impacts of the selected nanomaterials towards a range of cell types. Also on probing mechanisms in depth and connecting the observed impacts to the cellular localisation of the nanoparticles and to the nature of the protein (biomolecule) corona. Thus, year 3 gave us the opportunity to pull all of the individual elements and the extensive data obtained into a cohesive story of some of the routes by which nanoparticles interact with and impact on living systems.

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

* Weizmann Institute joined NanoInteract in Period 3.

Work Program

Throughout its three years, NanoInteract has sought 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 with the functional impacts of the various nanoparticles as a result of their presence in specific sub-cellular compartments. Thus, the overarching objective of NanoInteract was to create a firm scientific and technical basis for understanding and potential prediction of likely biological impacts of engineered nanoscale particles.

Fundamental to the project was the very considerable effort that was dedicated to the establishment of protocols and standards via which every step of the project was controlled as we sought to eliminate the factors that cause 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 3 and some highlights of the outcomes

The NanoInteract project has progressed well during the third year of activity, with increasing focus on probing deeply into the mechanisms of nanoparticle interaction with living systems, focusing on elucidating portals of entry of nanoparticles, how this affects the subsequent sub-cellular transport and localisation of the nanoparticles, and the connection between the final localisation of nanoparticles, the signalling and other functional impacts observed. The underpinning hypothesis of the NanoInteract project is that uptake is mediated by the nature of the protein (biomolecule) corona that forms around nanoparticles immediately upon contact with biological fluids, including cell culture media or the natural organic matter that is dissolved in river water. As such, very considerable effort has been devoted to understanding how these interactions affect the available nanoparticle dose and the dispersion evolution with time, under a range of biological conditions and over a variety of both short and long exposure durations.

A key aspect of the success of the NanoInteract project in the third year resulted from the successful implementation of the processes for controlling the nanoparticle systems used across all partners that were developed in the first period. This meant that each of the work packages could focus on assessing the impacts of the nanomaterials, safe in the knowledge that the nanoparticle dispersions and their evolution had been assessed under the appropriate exposure conditions and time-scales. In year 3 of the NanoInteract project, the investigations have focussed on several key classes of nanoparticles – titanium dioxide, silicon dioxide, cerium oxide, polystyrene, 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, and amine-modified polystyrene as this was the particle found to induce the most dramatic effects on cellular signalling and cellular function.

While it is hard to predict the long-term impact of the research performed within NanoInteract, it is clear that a major element of the impact will be an understanding of *how* to carry out durable, reproducible, collaborative research in the field of nanosafety assessment. It also seems likely that the nature and role of the 'protein corona' will clarify and may become a standard characterisation step for determining nanoparticle impacts. Indeed the first evidence of corona-induced uptake and functional impacts has emerged in Year 3 of the project, with some examples given below.

Interaction with biofluids determines nanoparticle dispersion characteristics

During year 3, a range of different methodologies have been applied in a time-resolved manner, to follow the lifetime of such biomolecular 'coronas' both *in situ* and isolated from the excess plasma.[1] Such particle-biomolecule complexes can be physically isolated from the surrounding medium, and studied in some detail, without altering their structure. For several nanomaterial types, we found that blood plasma-derived coronas are sufficiently long lived that they, rather than the nanomaterial surface, are likely to be what the cell sees.

An important manifestation of the interaction of nanoparticles with their surroundings is that the particles dispersed in biological fluids may no longer exist as simple monomeric particles with an associated protein corona. Thus, as shown in Figure 1, the hydrodynamic diameter of the nanoparticles increases in the presence of the protein corona, and populations of other species - dimer, trimer etc. appear in the size distribution curves as obtained by Differential Centrifugal Sedimentation (DCS). These particle multimer peaks are not resolved in dynamic light scattering, appearing instead as a broadening of the apparent size, suggesting that DCS is a much more appropriate technique for characterisation of nanoparticle size distributions *in situ* in biological fluids. Another manifestation of this is the case represented by 100 nm sulphonated polystyrene particles, where the particle monomers seem to disappear in the presence of plasma, suggesting that the nanoparticles partition into the intrinsic protein clusters, rather than associating with or drawing to their surface layers of proteins, illustrating the potential richness of the situation.

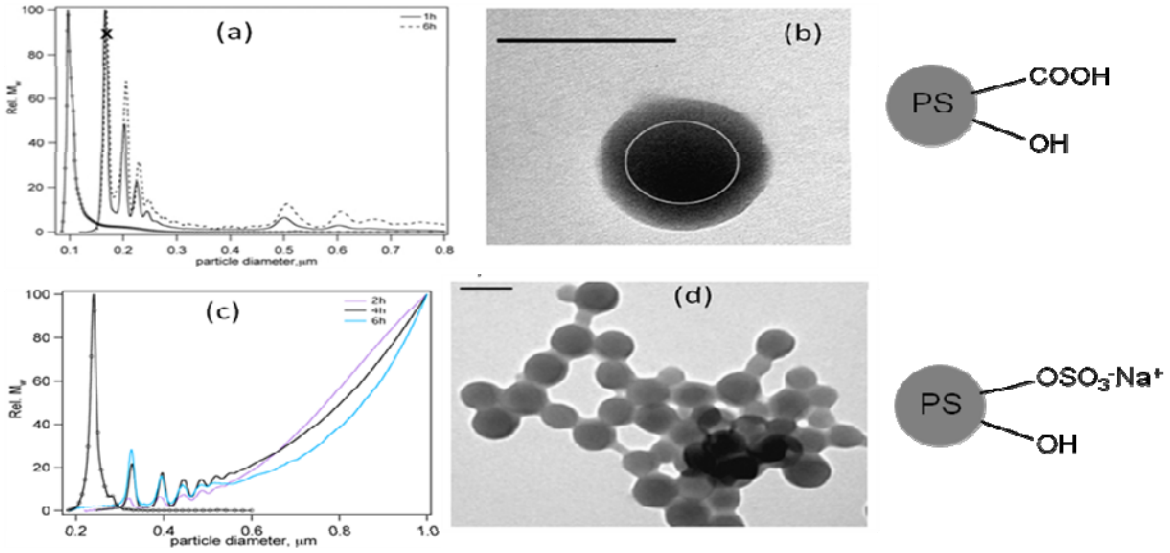

Figure 1. Example of the approach developed within NanoInteract to characterise the nature of nanoparticle dispersions in biofluids (e.g. plasma or cell culture medium supplemented with foetal calf serum) as a function of time. The method combines Differential Centrifugal Sedimentation to quantify the fraction of a dispersion that exists as monomeric, dimeric, trimeric and larger particle-proteins complexes, with TEM imaging of the nanoparticles with their protein coronas. Interestingly, in the second example shown (bottom image), it appears that the corona surrounds the particle clusters. In this case we believe that the protein clusters that exist naturally in plasma actually subsume the particles, rather than the nanoparticles drawing a corona to them, as is the case for most nanoparticles (top image). Redrawn from Walczyk *et al.*, 2010.[1]

The important consequence of this research is that although the composition of different particle-protein organizations in biological media may vary, if well designed dispersion protocols are used, one can achieve a high level of reproducibility of the populations of different particle-protein organizations (i.e. reproducible amounts of monomer, dimer, trimer, etc. and large complexes). This suggests the possibility of a rational and reproducible approach to studying and understanding bionanoparticle interactions with living organisms in future.

Manifestation of the evolution of nanoparticle dispersions in cell toxicity

Extensive studies with SiO_2 nanoaprticles from a range of different sources (including the Sigma Ludox nanoparticles, and particles synthesized via the Stöber method for the project by Glantreo Ltd. (a project partner) showed that in general, SiO_2 nanoparticles are not cytotoxic, although some exceptions related to use of stabilisers such as ethylene glycol did emerge. However, more detailed studies of specific end-points with silica particles shown to be non-cytotoxic at doses up to 400 µg / mL did show evidence of other, non-trivial biological responses.[2, 3]

For example, evidence of reproductive effects were observed with SiO_2 nanoparticles (no coatings, no impurities, amorphous) where *in vitro* inhibition of the differentiation of D3 cells was observed, as shown in Figure 2.[2] The extent of toxicity could not be directly associated with one single physicochemical property such as size, surface area or zeta potential, but is more likely related to a combination of these physicochemical properties which determine their interaction with surrounding particles, cells, proteins and other medium components, which affects the nanoparticle size distribution over the time-course of the stem cell differentiation study experiment (10 days, with fresh media containing nanoparticles added at day 5).

These results suggest that the widespread application of amorphous silica nanoparticles may not be without any health hazards. Long term exposure of high concentrations of these nanoparticles to humans could potentially result in particle accumulation and subsequently, induce acute or chronic toxicity, including to the embryo, suggesting that more research is needed on this aspect.

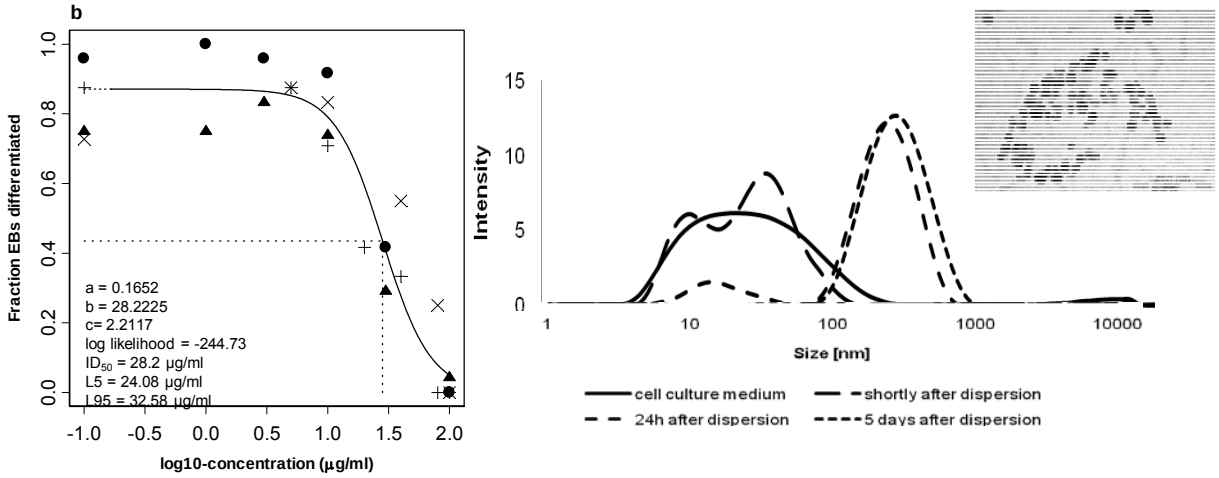

Figure 2. Reproductive toxicity: dose-response curve of the fraction of embryoid bodies differentiated into spontaneously contracting cardiomyocytes as a function of the nanoparticle concentration for nominally 30 nm SiO_2 nanoparticles. Dashed line represents the ID_{50} value. The symbols denote individual observations of four independent experiments. The curve on the right shows the actual size distribution of the particles over the time course of the experiment in the cell culture medium, with the inset showing a TEM of the dry nanoparticles.

Role of the nanoparticle protein corona in mediating nanoparticle uptake and impacts

A key result from Year 3 of NanoInteract is the confirmation of the central role played by the nanoparticle protein (biomolecule) corona in mediating the interaction of nanoparticles with living systems. Thus, we have shown that the nature of the protein corona determines the uptake behaviour (mechanism, kinetics, and localisation) as shown in Figure 3. Heat inactivation is a procedure commonly applied in cell culture that destroys the complement activity in the serum – and may alter the quality of the serum itself, thus affecting cell growth by destroying desirable active components or creating precipitates in the cell culture medium.[4] We found that the intracellular concentration (which is essentially the 'dose') of nanoparticles is affected by serum

heat inactivation, and complement depletion. The amount of adsorbed proteins in the long-lived (hard) corona is affected by heat inactivation, and correlates with nanoparticle uptake, with a more protein-rich corona leading to decreased particle uptake by cells. To what degree different cell-function effects (apart from *in vivo* immune and clearance effects) arise from the differently adsorbed proteins is not yet known.

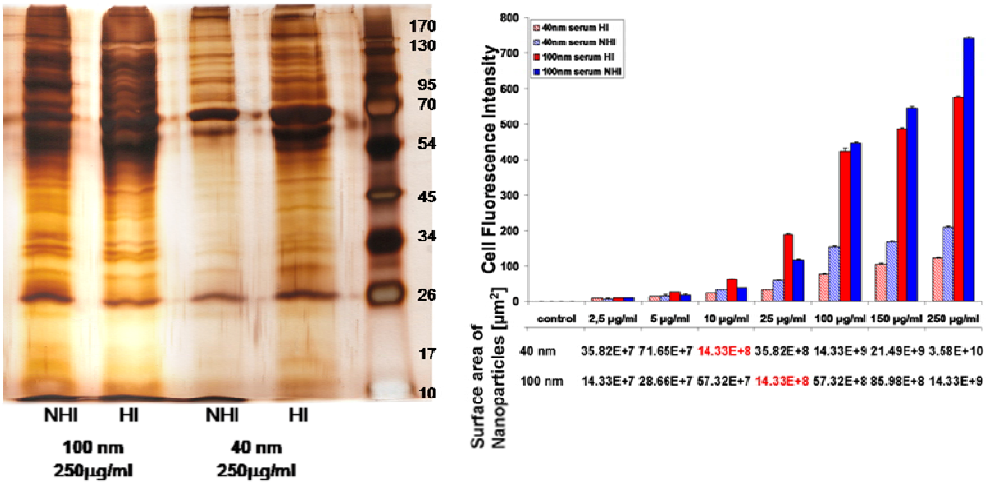

Figure 3. Clear example of nanoparticle corona composition and thickness influencing the kinetics of uptake of nanoparticles. Quite different uptake behaviours were observed for identical particles depending on whether the serum they were incubated with to form their corona had been heat inactivated (HI) or not heat inactivated (NHI). Redrawn from Lesniak *et al.*, 2010.[5]

Teaching and Training; 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 element of the NanoInteract project, both within the project, and externally in Year 3. Thus, elements of the protocols developed within NanoInteract have been utilised in the development of the NanoImpactNet training schools held in Lausanne in March 2009 (and planned for March 2010 and February 2011). An important output from the project is the set of documented protocols developed across the entire programme which are being formalised and transmuted into a training handbook and an associated training course.

Dissemination; Results are being disseminated as appropriate in the literature, with emphasis on high impact journals and papers from several partner institutions (over 23 of the NanoInteract publications involve two or more of the consortium partners). Additional dissemination routes involve conferences, involvement of key scientists in the working groups of ISO and the OECD on nanomaterials, and hand over of data and knowledge into other ongoing projects such as the Joint action 2010-2012 for safety of nanomaterials under the Public Health Program 2009. Significant advances from NanoInteract were communicated via the NanoImpactNet integrated conference in Lausanne in March 2009. A key output in Activity Period 3 has been the ongoing Chairing by P1 of the International Alliance for NanoEHS Harmonisation (IANH, www.nanoehsalliance.org). At the end of the third year of the project, over 50 articles have been published from the NanoInteract consortium, and an additional 15 additional manuscripts submitted or in final stages of preparation.

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