PUBLISHABLE EXECUTIVE SUMMARY

Development of an Integrated Platform for Nanoparticle Analysis to verify their possible toxicity and eco-toxicity

The DIPNA project was coordinated by Dr. Antonietta Gatti of CNISM-UNIMO (Italy), and the consortium was composed by:

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Aim of the DIPNA project was to provide knowledge about the impact of four different NPs (Cobalt, Gold, Cerium and Iron Oxide) applied in wet suspension and in dry state on different types of cells (human immortalised THP-1, HepG2, CaCo-2 and A549 cell lines, murine 3T3, human $CD34^+$-derived dendritic cells (DC), and human monocytes*)* to design assays and identify biomarkers of nanotoxicity. The project developed also technological solutions concerning the set-up of singlet NP/cell interaction with sensors to evaluate the effects, a device for an automatic evaluation of the toxicological impact of nanoparticles, and instruments for field analysis of nanopollution in occupational sites.
The project was divided into 6 Workpakages

WP1: Fabrication and characterisation of selected NPs
WP2: Nanotoxicology: NP-induced alterations of human defence cell physiology
WP3: Single particle impact on cells
WP4: Integrated platform for monitoring NP effects upon chronic and repeated exposure
WP5: Field validation and development platform
WP6: Coordination, management, training and public awareness

The DIPNA Project scientific (S) and technological (T) objectives were:

S1. Defining *in vitro* systems of interaction of engineered NPs with living cells, relevant to the assessment of immunotoxicity from acute, chronic and repeated exposure. Use of human professional (T cells, dendritic cells, monocytes) and non-professional defence cells (epithelial cells from lung, gut, liver) as models of innate and adaptive immunity, inflammation and angiogenesis; use of mouse fibroblasts as model of cytotoxicity, genotoxicity and carcinogenicity.

S2. Identifying the modes of NP-cell interaction: set up of one-to-one NP-cell systems (singlet interaction) to assess a dose-dependent *vs.* threshold effect.

S3. Applying the laboratory-developed cellular models to the field investigation.

T1. Development of *in vitro* simulation tests where a true **interaction** of cells with nanosized particles occurs (as opposed to aggregates or clusters). This objective is of key importance. Without the positive identification of the nanosized features of NPs during the cell assays, effects or lack of effects cannot be formally attributed to nanoparticles, as they can be due to aggregates or agglomerates. Internalisation *vs*. surface interaction has also to be assessed.

T2. Development of an *in vitro* system of single cell-to-NP interaction. This objective is highly innovative and can be realised only with the aid of advanced nanotechnologies. The detection system will reproduce the *in vivo* conditions, providing an accurate description of the real NP-cell interaction.

T3. Development of new sensors for detecting specific NP-induced biological reactivity, based on defined and validated sensible parameters. The test results will represent the basic knowledge necessary to the construction of new sensors. These will form the ideal platform for new advanced technologies.

T4. Assembly of a system for detecting and analysing NPs in industrial settings. The system will allow risk assessment within the working environment of nanotechnological laboratories or factories and could eventually contribute to set up prevention measures, thereby reducing risks to human health.

All objectives have been reached.

The results of the biological tests, carried out following the conditions of toxicological tests to assess the risks of molecules and ions, indicate that none of the NPs tested, either in dry or in wet conditions, affected the viability and capacity of proliferation of various cell types in culture. No signs of apoptosis were detected. The immunological biomarkers selected, such as cytokines, chemokines and TLR expression, were not affected in a dose-dependent fashion in the time frame of the *in vitro* tests.

A dose-dependent increase in the production of reactive oxygen species could be observed only for cobalt NPs. Also the expression of inflammation-related genes, such as IL-18Rα, TIR8 and caspase-1, was not modulated by NPs, either upon acute administration or when given in chronic or cumulative fashion over 15 days of cell culture. In the same conditions, silver NPs (used as control) likewise did not induce any relevant toxic effect nor could affect innate/inflammatory parameters.

The finding that only cobalt NPs seem to have toxic effects is explained as follows. Cobalt NPs can corrode in the culture medium and release cobalt ions. The toxicity of cobalt ions is well known. They are not in an equilibrium state and can readily combine with other ionic species present in the medium or in cells, and affect cell functions. Thus, toxicity of cobalt NPs can be indirectly due to the release of cobalt ions, rather than the consequence of NP-cell interaction. The identification of cobalt phosphate particles after chronic exposure of cells to cobalt NPs suggests that NP corrosion and ion release is indeed occurring.

Different systems were constructed to simulate singlet NP/cell interaction. Chips were built to monitor membrane changes by single cell impedance spectroscopy. A nanodispenser was devised for depositing NPs deposition in array format on cell culture supports. Microinjection techniques and Raman spectroscopy have been successfully combined with principal component analysis (PCA) to detect the single cell/(few) NPs interaction.

The singlet NP/cell interaction did not cause any measurable effect except for the appearance of signals in Raman spectroscopy. Such signals are due to phosphate groups present in NP-microinjected cells (but not in solvent-injected control cells), suggesting that microinjection of few NPs in a single cell affected the cellular phosphate metabolism.

An interesting system was developed for the repeated spraying of dry NPs in air and in a liquid medium containing cells. The system works very well in air but not in the medium.

An automated system was designed and constructed to measure the possible toxicity of NPs. The breadboard system consists of a small incubator with in-built controllers for CO_2, temperature and humidity, a fluidic system with pump and valves to transport reagents and samples, and two optical detection units, for optical absorption measurement and for refractive index measurement respectively. The system detects the proliferation of A549 cells (untreated *vs*. exposed to NPs) based on Alamar blue staining.

The results of DIPNA go beyond the objectives initially stated and provide several original information.

a- *In vitro* assays for the detection of NP effects on human cells

1. New reporter A459 cell lines were developed, based on expression of reporter genes under the control of different inflammatory cytokine promoters. These cells have been introduced successfully in all the assays for one-to-one NP/cell interaction and in biosensors.
2. NP acute effects (2-24 h) have been globally evaluated by applying transcriptomic analysis to A549 and CaCo-2 cells.
3. A new assay for acute massive exposure was developed through a lipofection method. An uptake of NPs 50% higher than normal was achieved without causing cell death. The assay allowed the identification of new chemical species (*e.g.*, iron-based and cobalt phosphate precipitates, after lipofection with cobalt NPs) both on and within cells. Similar new entities had been previously found also *in vivo* after cobalt NP implantation in the rat, and in human tumour samples.
4. A robust method for detection of NP effects after chronic and cumulative exposure was developed by using differentiated CaCo-2 cells (human gut epithelial mucosa). The method allows the integrated

detection of toxicity, epithelial layer permeability and transcytosis, and innate/inflammatory response after 15 days of exposure to NPs.

b- Instruments and methods for environmental NP detection

1. Gravimetric collectors and active sensors of NPs in occupational sites for off-line measures were constructed and used in nanotechnological laboratories and industries. Off-line evaluations of the occupational pollutants collected were carried out by Field Emission Gun Environmental Scanning Electron Microscopy coupled with an Energy Dispersive System for physical and chemical characterisation.
2. An aeromodel with a collector for air-dispersed NPs in the vicinity of chimneys of nanotechnological enterprises was constructed and applied.
3. A software for the automatic identification of NP size, morphology and chemical composition was implemented and adapted for FEG-ESEM observations of nanopollution in working places.
4. A data bank of nanopollution in some nanotechnological laboratories and factories was set up and some references of environmental normal pollution were identified.

c- Recommendations for nanotoxicologists or researchers

1. Dry NPs easily aggregate and form microsized particles that have a different behaviour compared to NPs. For this reason a control of the final state of NPs in the medium or within cells is mandatory in order to consider the results meaningful for nanotoxicity assessment.
2. Aggregation/sedimentation of NPs in liquid media and their adherence to vessel walls can profoundly alter their true concentration in solution. This may hamper the correct interpretation of toxicological results.
3. Nanotoxicological evaluation needs customised assays and handling procedures, different form the classical toxicological tests, in order to achieve reliable and relevant information. Only on such a basis effective strategies for knowledge-based development of the nanoindustry can be implemented that take into account workers' safety and public health protection.