Development of methodology for alternative testing strategies for the assessment of the toxicological profile of nanoparticles used in medical diagnostics. NanoTEST – EC FP7 project

Maria Dusinska, Lise Maria Fjellsbø, Eldbjorg Heimstad, Mikael Harju, Alena Bartonova, Lang Tran, Lucienne Juillerat-Jeanneret, Blanka Halamoda, Francelyne Marano, Sonja Boland, Margaret Saunders, Laura Cartwright, Sara Carreira, Susan Thawley, Maurice Whelan, Christoph Klein, Christos Housiadas, Katarina Volkovova, Jana Tulinska, Milan Beno, Katarina Sebekova, Lisbeth E. Knudsen, Tina Mose, José V. Castell, Maya R. Vilà, Lourdes Gombau, Mark Jepson, Giulio Pojana and Antonio Marcomini

1Norwegian Institute for Air Research, Norway
2Institute of Occupational Medicine, United Kingdom
3Centre Hospitalier Universitaire Vaudois, Switzerland
4University Paris Diderot Paris7, France
5University Hospitals Bristol NHS Foundation Trust, United Kingdom
6EC – Directorate General Joint Research Centre, Italy
7National Centre for Scientific Research Demokritos, Greece
8Slovak Medical University, Slovakia
9University of Copenhagen, Denmark
10Advanced In Vitro Cell Technologies SL, Spain
11Hospital Universitario La Fe. Valencia, Spain
12Department of Biochemistry, University of Bristol
13University Ca’ Foscari of Venice, Italy

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Abstract. Nanoparticles (NPs) have unique, potentially beneficial properties, but their possible impact on human health is still not known. The area of nanomedicine brings humans into direct contact with NPs and it is essential for both public confidence and the nanotech companies that appropriate risk assessments are undertaken in relation to health and safety. There is a pressing need to understand how engineered NPs can interact with the human body following exposure. The FP7 project NanoTEST (www.nanotest-fp7.eu) addresses these requirements in relation to the toxicological profile of NPs used in medical diagnostics.

1. Introduction
The overall aim of this project is to develop alternative testing strategies and high-throughput toxicity-testing protocols using in vitro and in silico methods which are essential for the risk assessment of these NPs. To be able to achieve this ambitious goal, the specific aims of NanoTEST are: a) to carry out a detailed characterization of selected NPs in order to define the major physico-chemical properties b) to study specific and nonspecific interactions of NP with molecules, cells and organs and to develop in vitro methods which can identify the toxicological potential of NPs; c) to validate in vitro findings in short-term in vivo models, to study manifestation of particle effects in animals and humans, and to assess individual susceptibility in the response to NPs; d) to perform both Structure-Activity modeling and physiologically-based pharmacokinetic (PBPK) modeling of NP; e) to adapt the most advanced and promising assays for high-throughput automated systems and to prepare them for validation by the European Centre for the Validation of Alternative Methods (ECVAM).

2. Selection of Nanoparticles and their characterization
The NanoTEST project will focus on the NPs used either in medical applications or as control or benchmark NPs: a) TiO2, as benchmark; b) paramagnetic metal oxides: Fe3O4, and rare earth oxides, which are applied for contrast enhancement as well as for cancer treatment in various biomedical applications; c) Metal fullerenes, which contain paramagnetic metals in the fullerene cage, proposed for use in MRI; d) polymeric materials utilised for the delivery of macromolecules (polyactic glycolic acid, PLGA, chitosan, hyaluronic acid), to be used as controls in order to ascertain the behaviour of nanoparticles at the biological level; e) Quantum dots (QD), used as alternatives for organic dyes for cellular labelling, imaging and tracking. The detailed description of main physico-chemical properties of selected NPs is an integral part of this project and selected reference NPs are being exhaustively characterised prior to use as exposure material for toxicological testings. All pertinent physico-chemical properties of concern, such as size distribution, shape, specific surface area, porosity, chemical composition, purity, impurities of concern, surface chemistry, surface charge, crystal structure, are being determined by applying various analytical techniques in order to ensure high quality of results. In addition, dispersion and stability of selected NPs in aqueous and biological media, as well as their interactions with culture media components, are being investigated. A broad range of different techniques, such as SEM, TEM, AFM, FFF, isotherm N2 absorption/desorption measurements (BET) for, ESR, NMR, UV/fluorescence measurements ICP-MS, HPLC-MS and GC-MS analysis are being employed to produce a well-characterised bank of NPs. At present two nanomagnetite NPs dispersions in water are being examined. Eventually, specific analytical protocols will be developed for the quantitative determination of the uptake and distribution of selected NPs in biological tissues after in vitro and in vivo exposure experiments.
3. Cellular models and biomarkers for toxicity testing \textit{in vitro} and \textit{ex vivo}

The mechanism of action of particles is likely to involve oxidative stress and inflammation. We will therefore focus on the cross-cutting areas of cellular toxicity, oxidative stress, inflammation, immunotoxicity, genotoxicity and related endpoints. We will define representative organs which can be used as predictors to evaluate in cellular models the biological response of exposure to selected medical NPs. We aim to propose relevant \textit{in vitro} toxicological models based on reference cell lines of these organs in order to identify reference biological markers and endpoints which can be used to test the possible toxicity of medical NPs. To identify these biomarkers, we will utilize different cell cultures (\textit{in vitro} models), organotypic cell culture and small fragments of organs (\textit{ex vivo} models), exposing them to NPs in clinical use. This will allow us to determine the mechanism of action and to predict the biological and physiological response of exposure to medical NPs and finally to evaluate the potential health hazards of NPs. Whilst it is not possible to consider all organs which may be potentially affected by exposure to medical NP, we intend to use a range of representative organs and to define representative cell lines of these organs. We will focus on cells derived from eight different biological target systems; blood, vascular system, liver, kidney, lung, placenta, digestive, renal and central nervous systems. All \textit{in vitro} studies will be co-ordinated and harmonized as much as possible by using the same batch of NPs, the same handling and safety procedures of NPs, the same exposure time, concentration of NPs, same protocols with similar endpoints, in order to be able to compare results and to evaluate the most relevant biomarkers and target cell types. The common draft safety protocols for handling nanoparticles are being prepared to be used by all partners. Development of \textit{in vitro} models using the cell lines and cells from different organs is in progress together with optimizing protocols for biomarkers of oxidative stress, inflammation, immunotoxicity, cellular toxicity and genotoxicity (Figure 1). Experimental protocols (Standard operating procedures) for biomarker detection to be used across the cell systems are under development.

An important cross-cutting area that NanoTEST will focus on is barrier transport of NPs. We will evaluate transport of NP across different barriers such as brain, lung, vascular, digestive system, kidney, placenta (Fig 1). Preliminary results from the placenta will be presented. The placenta is the interface between the maternal and foetal circulation during gestation and plays a very important role in maintenance of a healthy pregnancy and a successful delivery. Anything that interferes with placental function has the potential to have an adverse effect upon foetal development and may impact upon health in later life. Women may come into contact with nanoparticles through occupational,
environmental or medical exposure and this can happen at any stage of pregnancy including prior to them even being aware that they are pregnant. Therefore it is essential that we determine whether nanoparticles that are manufactured for use in medical procedures and which could be released accidentally pose any risk to the foetus. To determine this, we need to evaluate both transport characteristics and toxicity since whether a particle is able to transfer across the placental barrier will have a significant impact on where it accumulates and what cell types it may be in contact with. Ethically, this cannot be investigated in pregnant women and therefore NanoTEST will utilise two models to investigate these aspects: the ex vivo isolated human perfused placenta model and the in vitro human cell barrier model developed using the BeWo placental cell line. Transport experiments have commenced with a benchmark model particle (Fluoresbrite®) which show that, within the BeWo model, particles of 40-60 nm in diameter are capable of passing across an intact cell monolayer. Investigations are under way to clarify the mechanisms which are likely to involve endocytic pathways. Assays will be carried out in both systems to evaluate any toxicity resulting from exposure to the medical nanoparticles under investigation in this project.

4. In vivo animal –validation study
The toxicological profile of NPs in vitro will be validated in an experimental in vivo model. Rats will be used as experimental animals for sensitivity considerations. The route of exposure will be chosen according to the medical usage of the studied NP but most likely animals will be exposed by NP through intravenous injections. There will be a single exposure at the beginning of the study with subsequent sacrifice after 1, 2, 3 and 4 weeks. The effect of exposure to NP will be tested in the following organs: heart/aorta, lung, brain, blood, spleen, bone-marrow, liver, and kidney addressing the same biomarkers and endpoints as the in vitro study. Gross pathological and histopathological examinations will be undertaken according to OECD guidelines (425).

5. PBPK modelling and QSARS
One focus of NanoTEST is to identify descriptors relevant for the experimentally determined toxicity endpoints and suitable for modelling from the available data. The selected descriptors in addition to the experimentally determined physicochemical properties (size, surface area, solubility etc.) will be the starting point to develop Structure-Activity models using various statistical multivariate data analysis techniques. The computational algorithms which will be used for the physicochemical characterisation and derivation of descriptors important for the reactivity of the NPs will be chosen based on the particle type and composition, i.e. inorganic and organic structures. PBPK modelling will explore the possibility of formulating a simple compartment-based model to describe the biodistribution of NPs. Due to the limited amount of information available, only the salient features of partition between blood and different organ/tissues will be considered (i.e. small number of compartments). The work will use an existing compartment model as a starting point. In a second stage we will employ computational fluid-particle dynamics to analyze the behaviour of NPs in (i) the respiratory system and (ii) the cardiovascular system.

6. Database and development of standard operating procedures
The Consortium will generate an enormous amount of data to assess from all investigated systems. For this purpose a common NanoTEST (NAPIRAhub) database is under development and will be available for all partners. All the information obtained will be statistically evaluated and collected into a common database. Common approaches for handling, analyzing data and for comparison of data from different systems will be followed. Standard operating procedures for biomarker detection to be used across the cell system will be included in the databank. The data from the experiments will be collated, organised in a database and appropriate statistical analyses will be carried out to determine
the nature and extent of NP cell uptake, transport and toxicity over time using the various outcome measurements.

7. Automation assay
NanoTEST aims to develop automated assays to generate high quality data for input into modelling activities, and for future robust testing applications. A short-list of candidate assays for automation will be established. Priority will be given to protocols involving the study of principal mechanisms of action such as oxidative stress and inflammation. An evaluation study of the suitability for automation will be performed in context of the process flow of the selected SOPs and the capabilities of the automated testing platform. Detailed verification studies will be carried out to assess the performance of the assay using high quality reference substances and NPs. Once the candidate assays for automation are selected, an automated SOP, based on the established manual procedure, will be created. The complete automated assay will be verified with reference substances (e.g. positive controls).

8. Conclusion
A better understanding of how properties of NPs define their interactions with cells, tissues and organs in exposed humans is a considerable scientific challenge but one that must be addressed if there is to be safe and responsible use of biomedical NPs. This project will address many key elements in evaluation of NP uptake and exposure and toxicological effect using different biological systems and Structure-Activity models and PBFK modelling.

NanoTEST will integrate the investigation of toxicological properties and effects of NPs in several target systems by developing a battery of in vitro assays using cell cultures, organotypic cell culture and small organ fragments (ex vivo) derived from different biological systems; blood, vascular system, liver, kidney, lung, placenta, digestive, renal and central nervous systems. As NP action is likely to involve oxidative stress we will focus on the cross-cutting areas of inflammation, cellular toxicity, immunotoxicity, genotoxicity and related endpoints. Following development of Standard Operating Procedures and generation of a common database and in parallel with in silico assays (QSAR, PBPK modelling), NanoTEST will evaluate toxic effects and interactions of NPs used in nanomedicine. There are a number of different NP characteristics which will influence transport and toxicity including size, surface area, coating and charge. With the use of a suitable panel of NPs of the highest purity we will determine how these characteristics relate to possible adverse health effects. We aim to develop a battery of in vitro and in silico assays to evaluate toxic effect of NPs used in nanomedicine. Results will be validated in an experimental ethically approved experimental in vivo model. The most advanced and standardised techniques will be adapted for automation and prepared for validation by ECVAM. Finally, we will propose recommendations for evaluating the potential risks associated with new medical NP, which will be communicated to the scientific and industrial community.

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