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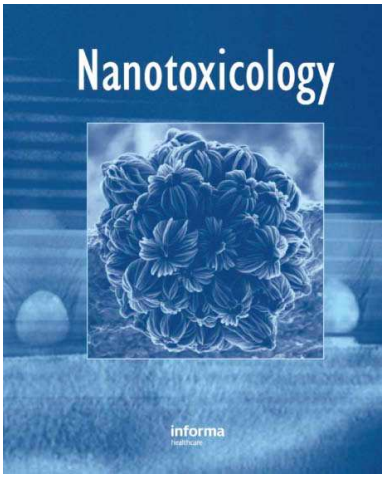

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Biological impact assessment of nanomaterial used in nanomedicine. Introduction to the NanoTEST project

Lucienne Juillerat, Lise Marie Fjellesbo, Maria Dusinska, Andrew Richard Collins, Richard Handy, Micheal Riediker

doi:10.3109/17435390.2013.826743

Abstract

Therapeutic nanoparticles (NPs) are used in nanomedicine as drug carriers or imaging agents, providing increased selectivity/specificity for diseased tissues. The first NPs in nanomedicine were developed to increase the efficacy of known drugs displaying dose-limiting toxicity and poor bioavailability, and to enhance disease detection. Nanotechnologies have gained much interest owing to their huge potential for applications in industry and medicine. It is necessary to ensure and control the biocompatibility of the components of therapeutic NPs to guarantee that intrinsic toxicity does not overtake the benefits. As well as monitoring their toxicity in vitro, in vivo and in silico, it is also necessary to understand their distribution in the human body, their biodegradation and excretion routes, and dispersion in the environment. Therefore, a deep understanding of their interactions with living tissues and of their possible effects in the human (and animal) body is required for the safe use of nanoparticulate formulations. Obtaining this information was the main aim of the NanoTEST project, and the goals of the reports collected together in this special issue are to summarize the observations and results obtained by the participating research teams, and to provide methodological tools for evaluating the biological impact of NPs.

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Biological impact assessment of nanomaterial used in nanomedicine. Introduction to the NanoTEST project

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www.nanotest-fp7.eu

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Key words: nanoparticles**,** therapeutic nanomaterials, risk assessment, biocompatibility

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Abstract

Therapeutic nanoparticles (NPs) are used in nanomedicine as drug carriers or imaging agents, providing increased selectivity/specificity for diseased tissues. The first NPs in nanomedicine were developed to increase the efficacy of known drugs displaying dose-limiting toxicity and poor bioavailability, and to enhance disease detection. Nanotechnologies have gained much interest owing to their huge potential for applications in industry and medicine. It is necessary to ensure and control the biocompatibility of the components of therapeutic NPs to guarantee that intrinsic toxicity does not overtake the benefits. As well as monitoring their toxicity *in vitro*, *in vivo* and *in silico*, it is also necessary to understand their distribution in the human body, their biodegradation and excretion routes, and dispersion in the environment. Therefore, a deep understanding of their interactions with living tissues and of their possible effects in the human (and animal) body is required for the safe use of nanoparticulate formulations. Obtaining this information was the main aim of the NanoTEST project, and the goals of the reports collected together in this special issue are to summarize the observations and results obtained by the participating research teams, and to provide methodological tools for evaluating the biological impact of NPs.

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Introduction: therapeutic nanoparticles in human medicine

Progress in material science and physics has led to novel medical applications based on the intrinsic properties of nanomaterials. The term 'nanomaterials' covers materials with one, two or three dimensions in the nanoscale while nanoparticles (NP) are usually defined as systems for which all three dimensions are between 1-100 nm. The European Commission recently adopted the following definition: "*A natural, incidential or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1-100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50% may be replaced by a threshold between 1 and 50%.* [European Comission. Nanomaterials. http://ec.europa.eu/environment/chemicals/nanotech/index.htm]." However, no clear consensus has been established for a definition of NPs/nanomaterials among scientists (Kreyling et al. 2010).]. Different fields of nanotechnology apply slightly different definitions, especially concerning the size limit. The term nanoparticle in the field of nanomedicine encompasses particles with dimensions up to 1000 nm (Schütz et al. 2013a). NPs include solid core NPs, usually coated with polymers for colloidal stability and biocompatibility, whose thickness and charge can depend on environmental parameters and which cannot easily deform; and polymeric NPs, formed of polymer-copolymer systems, whose characteristics depend dramatically on environmental factors such as temperature, pH, ionic strength or medium quality, and which can easily deform to pass certain biological and physical barriers (Godin et al. 2010; Schütz et al. 2013b).

Thus molecules and nanoscale materials are manipulated to produce nanostructures that can interact with human cells, offering a range of new solutions for diagnosis and smart therapies. The 1st generation of therapeutic nanovectors relied on size effects, taking advantage of the enhanced retention and permeability (EPR) and avoidance of cell uptake; the 2nd generation nanovectors presently under evaluation rely on improved nanovector surfaces, and the 3rd generation will rely on smart nanovectors bearing

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reconnaissance molecules aimed at defined cell types for improved selectivity and cell uptake (Juillerat-Jeannerat, 2006; Godin et al. 2011). Applications of nanoparticulate systems in medicine are aimed at improving diagnostic processes, increasing the efficacy of drugs known to have dose-limiting toxicity and/or poor bioavailability and biological stability, and improving the selectivity of drug delivery and of drug uptake by tissues (Schütz et al. 2013a,b).

The first marketed therapeutic nanomaterial, approved in 1990, was Adagen® - a PEGylated -liposomal-doxorubicin. Since then more than 40 nanoparticulate systems have been approved (Boulaiz et al. 2011; Schütz et al. 2013a; 2013b), mainly in the field of cancer treatment and predominantly represented by liposomal and micellar forms of widely used but poorly bioavailable anti-cancer agents. A variety of chemotherapeutic NPs are presently under pre-clinical development, in clinical use, or under advanced clinical trials (Juillerat-Jeanneret, 2006; Schütz et al., 2013a; Schütz et al., 2013b) (Table 1).

After drug delivery, the most represented area of application is diagnostic imaging, including in most cases iron oxide-based NPs for magnetic resonance imaging (MRI) and gold NPs (Pablico-Lansigan et al. 2013). The third application is the preparation of therapeutic agents as nanosuspension (Patel et al. 2011; Figure 1). Therapeutic NPs have evolved to include, for example, combination of imaging and therapy for diagnosis and thermal ablation of lesions, the so-called "*theranostics*" (Lammers et al. 2011).

The choice of the materials composing therapeutic NPs depends not simply on biochemical/physical characteristics but also on whether biodegradation of the carrier is required, whether tissue reaction to the NPs can be avoided, and whether increased efficacy of the therapy can be achieved. Nanovectors are designed to afford protection of drugs against degradation (Godin et al. 2010; Godin et al. 2011), but controlled drug release is difficult to achieve and the interactions with living cells and tissues are poorly defined (Naahidi et al. 2013). If the biocompatibility of NPs cannot be ensured, potentially advantageous properties of therapeutic NPs may produce toxicological problems. It is important to identify the properties, to understand the mechanisms by which NPs interact with living systems and thus to understand exposure, hazards and possible risks of therapeutic NPs (Teow et al. 2011; Lai 2012; Klaine et

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al. 2012).

Addressing toxicity of therapeutic nanoparticles – the NanoTEST project

The rapid growth in the use of nanomaterials in medicine has led to a concern about possible health risks. There is a serious lack of information available to predict health effects due to nanomaterial exposure (Pautler and Brenner 2010); in a regulatory context, nanoscale materials are still mostly treated in the same way as conventional chemicals and there is no consensus or clear nano-specific guideline for their regulation (Dusinska et al. 2009; 2011; 2012; 2013; Schrand et al. 2012). However, several scientific opinions (SCCS 2012; SCENIHR 2007; SCENIHR 2009; Scientific committees 2013), guidelines and specific European regulations and OECD guidelines such as for cosmetics (EU Directive 2009; OECD 2012), food contact materials (EFSA 2009; EFSA 2011; OECD 2012), medical devices (OECD 2012; SCENIHR 2012) as well as the US Food and Drug Administration (FDA) regulations specifically address nanomaterials (FDA 2010; Duvall 2012).

The NanoTEST project (*www.nanotest-fp7.eu*) was one of the initiatives set up by European Commission to fill the knowledge gaps in this area, by studying the interactions of representative therapeutic nanomaterials with living cells, then developing and validating appropriate high-throughput toxicity testing protocols using *in vitro* models. Keeping in mind the 3 Rs (refine, reduce, replace animal testing), the testing strategy should reduce *in vivo* experiments. To achieve this, a comparison of *in vitro* and *in silico* with *in vivo* results is an important and critical aspect of the validation of *in vitro* and *in silico* methods. One of the main problems encountered in testing NPs for possible human hazard is the lack of appropriate standard protocols.

The NanoTEST project started in April 2008. During the subsequent four years, twelve partne institutionrs were involved in the project, bringing together top scientists from ten European countries. Harmonisation has been the key element at every step, and we have used standard protocols and the same data templates in order to be able to compare results, as well as using NPs from same batch. NanoTEST consists of six

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work packages (WPs): Characterization, *In vitro* studies, *in vivo* studies, (Q)SAR and PBPK modelling, Dissemination and Management (Figure 2). Close collaboration between the WPs has been of great importance in achieving our goals.

A representative selection of nanomaterials was investigated. Commercial nanomaterials currently or soon to be applied in human medicine were chosen; Nanomagnetite coated with Na-oleate (OC-Fe_3O_4); Nanomagnetite uncoated (UC-Fe_3O_4), Red (Rhodamine) Fluorescent nanosilica 25nm and 50 nm (Fl-25 SiO_2 and Fl-50 SiO_2), and Polylactic glycolic acid polyethylene oxide (PLGA-PEO). Titanium dioxide (TiO_2 NP) was used as benchmark material.

Physicochemical and biological characteristics of nanomaterials

There is a general agreement that not only one, but several characteristics may influence the toxicity of NPs (Bouwmeester et al. 2011). The specific features and properties of NPs which differ from those of conventional chemicals include size, size distribution, surface, shape, chemical composition, bioavailability, solubility, agglomeration, dissociation and adsorption of biological molecules, all of which may have an impact on the ultimate toxicity of the NPs (Warheit et al., 2007; Handy et al. 2008a). NPs, including therapeutic NPs, have unique properties probably resulting from their high surface-to-volume ratio and surface reactivity (Stone et al., 2010). Within NanoTEST, the incorporation of physicochemical data on nanomaterials into a knowledge base was crucially important in order to investigate their potential biological effects. The selected NPs were exhaustively characterized prior to being applied as exposure material in toxicological tests, both as stock solutions and in the medium to be used for the specific cells/experiment. It was necessary to obtain comprehensive characterisations in addition to sound toxicological data in order to develop Structure-Activity models for toxicity predictions. Our aim was to provide a framework for assigning hazard categories to nanomaterials on the basis of screening tests and predictive modelling.

Accurate and precise methods – both analytical (for characterisation of therapeutic NPs) and biological

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(for efficacy and/or safety assessment) – as well as appropriate *in vitro* and *in vivo* models must be developed and validated in order to analyse factors such as solubility, biodegradability and dissolution of NPs in biological media, their interactions with living organisms, their reactivity and uptake by cells and tissues. Effects of therapeutic NPs depend on their potential for biological membrane penetration and their (intra-)cellular targets. Some information is already available; for solid-core therapeutic NPs, size of the core and the physicochemical characteristics of the surface polymers are the main determinants for tissue localization and cell effects of these nanovectors. The therapeutic efficacy and biological effects depend on the chemical components of these NPs, their solubility in a biological environment and their intra-cellular localization. Transport of NPs across biological barriers (cell membranes and cell-layer barriers) is very specific/selective and is as yet poorly understood (Handy et al 2008b). Some NPs are designed to overcome barriers, such as the blood brain barrier, but do not need to enter cells, while others need to enter cells to give the biological effects.

The physico-chemical properties of NPs and the potential changes to NPs during absorption, distribution and metabolism in wildlife (Handy et al. 2008b) will equally affect the toxicity and fate of therapeutic NPs in the environment.

Polymer-copolymer therapeutic NPs are very difficult to locate in living tissue. Some polymers and/or their degradation and biotransformation by-products may be cytotoxic over the long-term. However, the lack of clear clinical parameters as targets to monitor NP-related toxicity still limits the use of pharmacovigilance monitoring to collect data on the long term toxicity of NPs. Very little knowledge exists concerning the consequences of the uptake by cells or tissues of nanovectors, or the mechanisms of their degradation and elimination by cells, tissues and the whole body, even for the nanovectors in clinical use.

Therapeutic NPs are different from NPs in ambient air pollution. However, the latter can inform about some of the characteristics that can lead to elevated hazards. Ambient particles are associated with a clearly increased risk for cardiovascular and pulmonary morbidity and mortality and the creation of reactive oxygen species (ROS) has repeatedly been proposed to be centrally involved in this response (Brook et al.

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2010). A recent study of workers exposed to diesel exhaust suggested that functional groups such as reactive carbonyls promoted oxidative stress (Setyan et al. 2010). Thus, a potential strategy could be to evaluate the types of functional groups that are formed during the degradation of nanomaterials, and to compare them with a (yet to be established) library listing the oxidative stress potential of a wide range of functional groups. This would allow the identification of potentially critical materials even before they are created and tested in real cells and animals.

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Nanoparticles in the human body; uptake, distribution and excretion

Basic questions concerning nanomaterials in the human body have yet to be adequately addressed including whether therapeutic NPs penetrate cells and tissues; how and where are they distributed inside the body; as well as how they are bio-degraded and excreted.

The specific characteristics of nanomaterials compared to standard chemicals mean that it is not possible to transfer, without verification, the knowledge acquired with standard chemicals to nanoparticulate systems (Casals et al. 2010; Monopoli et al. 2011; Lundqvist et al. 2011; Milani et al. 2012).

For drug delivery, NOs are administered orally, by inhalation, transdermally or parenterally (e.g. intravenous, intramuscular or subcutaneous). However, the main route of bio-distribution of therapeutic NPs in humans is the blood circulation, and it is necessary to consider the behaviour of these NPs in this organ. At the site of application and in the blood, a process called opsonization adds biomolecules onto the NP surface, forming a corona (casals et al. 2010; Lundqvist et al. 2011; Milani et al. 2012), the nature of which depends both on the surface characteritics of the NP and on the characteristics of the tissue. This coating of biomolecules will then determine the bio-distribution of the NPs in the patient, their behaviour in the body and the long-term consequences of their application.

The properties of NPs and their reactivity may change when entering the human body, through surface functionalization by biological components, or through bio-degradation. NPs can be deposited in the different organs through blood and can accumulate in secondary target organs such as liver, spleen, brain,

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kidney and heart although the exact amount is subject to debate (Oberdörster et al. 2004; Mills et al. 2006). Following access to the systemic circulation, particle half-life would normally be determined primarily by kidney elimination and ability to escape phagocytosis by the reticulo-endothelial system (liver, spleen, lymph nodes). For example, 36% silica particles (Ø 150 nm) after administration into rats' circulatory system were excreted with urine after four days. The remaining particles were accumulated in the kidneys and lungs, probably in the lung air sacs and kidney glomerulus (Borak et al. 2012). Silver nanoparticles (20 and 200nm) were translocated from the blood to the main organs, mainly to liver and kidney (Dziendzikowska et al. 2012). Surface-modified PLGA NP were localized within the tissue parenchyma (brain, kidney, liver, spleen and lung) (Tosi et al. 2010). NP characteristics such as size, surface charge and coatings are likely to affect trapping or targeting of tissues (Borm & Müller-Schulte 2006, Nemmar et al 2006, Hirn et al. 2011).

We are only beginning to understand the dynamics and bioreactivity of NPs in complex biochemical matrices. Following their use and bio-transformation in humans and their excretion, their fate and behaviour in the environment are equally complex since these NPs have been biochemically modified by their passage through the human body, a process for which presently information is almost non-existent (Figure 3). The development of a life cycle-based evaluation of the impact of therapeutic NPs implies the development and validation of methods and tools for assessing the relationship between the characteristics of the NPs, their bio-distribution and bio-transformation in the human body, and environmental effects. The fate and toxicity issues presented by nanomaterials are highly important questions that have not yet been resolved, either scientifically or from a regulatory perspective.

Toxicity testing

In vivo pre-validation

In vitro screening is increasingly used as part of hazard assessment. However, for understanding and interpreting results, *in vivo* validations are crucial. Experiments on animals were an integral part of the

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NanoTEST project (Dusinska et al. 2009; Sebekova et al. 2013; Volkovova et al. 2013). Female rats were used as experimental animals for sensitivity considerations and the possibility of examining fetal exposure. The rats were exposed with three doses of NPs by intravenous injections. There was a single exposure at the beginning of the study with subsequent sacrifice after 1, 2, 3 and 4 weeks. The effects of exposure to NPs were studied in the heart/aorta, liver, lung, brain, blood, spleen, bone marrow. Gross pathological and histopathological examinations were made according to the guidelines of OECD (433). In parallel with the *in vitro* screening, the following biomarkers were followed; cardiotoxicity, hepatotoxicity, nephrotoxicity, neurotoxicity, damage in lung tissue cells, genotoxicity, oxidative stress, immunotoxicity and routine basic haematological examinations undertaken at sacrifice.

The state-of-art when the project started did not provide information about standard procedures used for testing nanomaterials, and so the information gained from *in vivo* experiments is an important contribution for assessing nanomaterial toxicity. Strategically it was most important to compare and validate the *in vitro* results with *in vivo*. It would be too ambitious to compare all the endpoints mentioned above, and the focus was therefore to assess the effects of NPs on genotoxic and immune response with the aim to compare results of *in vitro* findings with *in vivo* findings in rats. A panel of immune biomarkers applicable in both *in vitro* and *in vivo* models was used for the assessment of possible immunotoxic effects of NPs. The same assays were performed *in vitro* in human peripheral blood and in peripheral blood of rats exposed *in vivo* to TiO_2 and OC-Fe_3O_4 NPs.

In vitro screening

Dependent on the route of exposure, various organs, tissues and cells can be exposed to NPs. To reduce amount of tests, certain organs were selected and representative cell lines derived from these organs were defined. These were chosen on the basis of the most likely cells to be affected, depending on the exposure route. In nanomedicine the most likely route is intravenous injection, where first to be affected are the plasma and circulating cells, the cells of the vascular wall (e.g. brain-derived endothelial cells), followed

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by the liver, the lung, the placenta, the brain and the kidneys. Inhalation will affect the epithelial cells of the bronchi and the alveolae and immune cells of the lung surface. Oral exposure will affect cells of the stomach and the colon.

As the activity of NPs is likely to involve specific toxicity endpoints, the project focused on the cross-cutting areas of cellular toxicity, uptake, transport through barriers, oxidative stress, inflammation, immunotoxicity and genotoxicity. An illustration of the structure of *in vitro* screening is given in Figure 2. The main objectives of the *in vitro* screening were; to evaluate the impact on specific cell functions of exposing cells or tissues of different phenotypes to NPs of various chemical composition, size and surface characteristics; and to define objective markers of cell reactions elicited with the selected NPs, which would serve as reference markers when studying novel nanomaterials. A better understanding of how properties of NPs determine their interactions with cells, tissues and organs in exposed humans and animals is a considerable scientific challenge but one that must be addressed if there is to be safe and responsible use of biomedical NPs. This process involved the development and adaptation of reliable and standardized *in vitro* assays and protocols to ensure that the potential risks of NPs are properly assessed. These assays and protocols were standardised across the Consortium through the use of the same NP dose range, exposure time-points and extent of cell confluency at time of exposure. Results from the *in vitro* screening were used to determine the assays most suitable for automation and to refine the in vivo experiments required.

Modelling

Using computerized tools for assessing hazard and risk may be valuable for screening and gives an opportunity to save time and resources compared with experimental studies. These approaches must be validated, adequately explained and made accessible to peer-review to be of any value for risk assessment and regulatory use (Nel et al. 2012). Structure-activity modelling is based on the assumption that similar chemical structures exhibit similar biological activities, thus enabling development of mathematical

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models relating chemical structure to activity. The use of Structure-Activity models, such as (Q)SARs and chemical category approaches, are well-established approaches for predicting the properties and activities of chemicals; however, research on descriptors which encode the specific reactivity of NPs is still scarce. Obviously, the development of Structure-Activity models requires a whole knowledge of properties affecting the toxicity of NPs and very few studies have attempted to develop these models for NPs (Bosi et al. 2004). In NanoTEST one of the aims was therefore to identify descriptors relevant for the experimentally determined toxicitity endpoints and suitable for modelling from the available data. For that purpose, expert knowledge as well as different "variable selection" statistical approaches were used. The selected descriptors in addition to the experimentally determined physicochemical properties (size, surface area, solubility etc.) were the starting point to develop Structure-Activity models using various statistical multivariate data analysis techniques.

Extrapolation modelling such as Physiologically Based Pharmacokinetic (PBPK) can be used to equate tissue-medium concentrations from toxicity testing with tissue doses expected in humans (Nel et al. 2012). The fate of nanomaterials in the body was studied using PBPK modelling approaches, by implementing two levels of refinement, corresponding to lumped parameter models and advanced mechanistic (distributed parameter) models. The former consisted of a simple compartment-based modelling approach and the latter model was based on computational fluid-particle dynamics to simulate detailed deposition in the respiratory or the cardiovascular system. The use of computational methods for deposition and behaviour in the lungs or the cardiovascular system can replace *in vivo* toxicokinetic tests and therefore significantly contribute to alternative testing methods.

Impact assessment

The tools used in assessing the risks that chemicals in general pose to people and the environment include toxicology, molecular biology, chemistry, high-throughput technologies and computer science; they are applied ultimately to develop predictive models, and the toxicological evaluation is used by regulatory

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agencies to provide recommendations for health and safety. However, while the technologies have been developed and validated for many years for chemicals, the process is much newer with NPs, and there is a need to develop evaluation processes and validation protocols. The factors to consider when assessing the use of NPs in nanomedicine include their size, exposure scheme, dose, the population involved and the length of time of exposure (chronic versus acute exposure). With therapeutic NPs in particular, there are substantial gaps in our knowledge of the behaviour of these NPs in humans (Figure 4), and the life-cycle evaluation of their impact is far from complete. The difficulty facing the regulatory agencies is the need to regulate emerging technologies at the same time as research is progressing, in the absence of a full scientific understanding of which parameters to control and review.

Novel technologies based on NPs will clearly benefit patients and will have a direct impact on health and the quality of life in the future. They have the potential to improve health monitoring, diagnosis, treatment, and can contribute to the repair of tissues and organs to arrest the progression of disease. It is also expected that nanomedicine will play a role in "personalized medicine". But the beneficial effects must not be at the price of increased risk for the patients; the new technologies must be safe and sustainable, for human health and the environment.

Life cycle impact assessment is a formalised concept for evaluating the relative biological and environmental effects of a product in general (ISO 2006a; ISP 2006b). Its application to nanomaterial is under validation. All stages of a nanoproduct life cycle – the production, transport, use and final disposal of the product(s) – need to be considered (Som et al. 2010), taking into account the by-products of their bio-transformation in the human body. A thorough impact assessment will facilitate the process of decision making by industry, research institutions and regulatory agencies. It will also help to identify gaps in knowledge and to prioritise topics for further research.

However, the experimental approaches to be used must first be defined. Presently it is not clear how *in vitro* data can predict *in vivo* effects. It is necessary to evaluate whether *in vitro* toxicological approaches, with their limitations such as the lack of information on the uptake and distribution of NPs *in vivo*, are

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relevant. *In vitro* studies can provide information concerning potential toxicity and dose dependency, and fundamental knowledge on biological targets and effects. The importance of particle characterization, not only as prepared, but also as bio-modified in biological matrices has also to be emphasised.

The knowledge gaps identified include a lack of knowledge on appropriate/optimal metrology, biological mechanisms and modes of action, toxico-kinetics (biopersistence), realistic exposure levels in relation to increasingly sensitive methods to detect effects of NPs, standardised testing protocols, transport (and/or indirect signalling) across biological barriers, and quantitative *in vitro-in vivo* extrapolation of the effects of NPs. NPs often display different chemical, physical, and biological characteristics from those of the bulk form of the same substance, highlighting the need for specific nano-policies (Baun and Hansen 2008). However, for many NPs, production volumes (by mass) are well below the threshold necessitating the provision of data relating to health and safety (Malkiewicz et al. 2009).

The current guidelines for testing medicines from the EU medicines agency do not require any nano-specific protocols, raising concerns that hazards can be missed or overestimated. However, a focused review of nanomedical patents and published literature (Schütz et al. 2012) to assess the incidence and type of safety data being reported for nanomedicines under development showed an encouraging level of toxicity evaluation.

Just as with the toxicity testing of therapeutic NPs described above, there is also a concern about monitoring public health in the environment and there is a lack of agreed protocols to measure human exposure or effect (hazard) in non-clinical settings.

Clinical Trial Authorisations and NanoTEST

Current regulatory procedures for approving new medicines (and those for medical devices) also apply to nanomaterials. The overarching process of conducting a clinical trial with a medicine intended for human use is covered by the Clinical Trials Directive (DIRECTIVE 2001/20/EC) which sets out the

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implementation of good clinical practice for such trials, and various codes relating to medicinal products for humans (e.g., DIRECTIVE 2004/27/EC). In addition regulation EC number 726/2004 lays down the procedure for the authorization and supervision of medicinal products and this involved establishing the European Medicines Agency with some oversight of national level authorities within Europe (REGULATION (EC) No 26/2004). Clearly, these regulations set out a well defined process which must be followed, and (until scientific evidence shows we need something different) these regulations apply just as equally to nanomedicines as the fundamental purpose of the regulations is the same for all medicines. Outside the European Union, regulations tend to be set at national level. For example, in the United States the FDA Code of Federal Regulations 21 sets out the provisions for drugs intended for human use (FDA, 2012); but these national level regulations do consider the principle of international harmonization when setting their rules. There are some key foundations on which the approval of any new medicine is based; (i) demonstrating that the new product works and is effective for its intended clinical use; (ii) if it is replacing an existing medicine the product should be better or more effective than the existing product, and (iii) that the new product is safe, or safer than the old one. NanoTEST and the mammalian research in the EU nanosafety cluster is contributing data mainly in the pre-clinical phase where proof of principle (efficacy and mode of action) and safety should be established for the candidate medicine. The overall clinical trials process including the drug registration and pharmacovigilence during its initial use is very expensive (e.g., typically 10-15 years from idea to medicine, circa 350 million Euros). This represents a big financial investment for the drug company and the *in vitro* and *in silico* modelling in the early stages is therefore crucial to both the scientific and financial investment into a prospective medicine. Making decisions on drug development requires confidence in the methodology as well as results of the *in silico* and *in vitro* work. The NanoTEST project goes some considerable way to providing the evidence-base that shows that these approaches can and do work for therapeutic NPs.

The next step for the drug company is to extrapolate from *in vitro* to *in vivo* (discussed above) and from a regulatory perspective the routine requirement is to include at least two relevant species where the test

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animals are expressing the target receptors for the drug of interest. The latter is problematic for nanomaterials in that the material may not be intended for the classic receptor-mediated drug response, or there may be concerns that the NP is immunogenic (Dobrovolskaia and McNeil, 2007). The regulations also require that the route of uptake, frequency of dosing, formulation, concentration and administration site must be related to the expected use in humans, and in NanoTEST for example, experiments are included on intravenous injections of NPs.

After successful animal studies, the prospective medicine is evaluated for safety in a small group of healthy human volunteers. For therapeutic NPs the problems of jumping the species barrier to man are the same as for conventional medicines, including; the need to account for differences in body sizes/blood volumes between laboratory rodents, marmosets, and humans; correcting for mass effects on drug metabolism with mass specific metabolic rates; for oral medicines dealing with the changes in gut chemistry/nutrition as one moves from animals to man, as well as differences in blood chemistry between species. For conventional medicines these extrapolations across species are routine and the industry makes use of look-up tables for blood volume differences and distribution times of soluble substances, species specific metabolic rates, etc. However, these data in many cases do not exist for NPs and (for example) given the aggregation properties of NPs we should not assume that standard look-up tables of blood volume will tell us anything about the predicted concentration in humans – just multiplying up the blood volumes across species is not a safe assumption for NPs. NanoTEST has incidentally captured some of this required data during *in vivo* experiments. A complete histopathology on the animals is also required before a first dose into man can be given, and some of the data in NanoTEST show organ pathologies that are also well known for other chemicals, adding to confidence in these kinds of data for the industry.

The regulations also require that the safety trials in human volunteers (i.e., phase I studies) demonstrate that the medicine has no or limited adverse effects on patients (no SAE, serious adverse event, or serious adverse drug reaction, ADR), and gives measurable dynamics (e.g., in the blood) that enable practical considerations such as likely margins of safety for prescribing errors/accidental overdose in the real world

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(large therapeutic index). The problem for nanomaterials is to know precisely what to put in place for patient care should the first dose into man go wrong. The usual routine of monitoring vital signs, having a team on stand-by to deal with cardiac arrest, etc. (i.e., the obvious clinical obligations to ensure volunteer survival) will always be in place, but anticipating the unexpected is the real challenge for NPs and there are regulations that say "suspected unexpected adverse reactions" should be reported to the authorities. Typically, unexpected adverse reactions can be inferred from the fine details in the pre-clinical data, often relying on very experienced staff piecing together small details from animal and *in vitro* studies as possible adverse effects in the first human study. So for example, if the animal histopathology showed some changes in the anatomy of the thyroid, then it might be worth measuring metabolic rate or circulating thyroxine levels in humans as a potential adverse effect that may appear, even if it not related to the mode of action of the NP. It is the absence of such logic(s) and detailed measurements in the patient that usually leads to unexpected catastrophic outcomes. Thus, both new ways of thinking and comprehensive data sets need to be developed for NPs. NanoTEST provides relevant data from pre-clinical investigations, but we have yet to detail the cross-talk between body systems for NPs in order to look for the unexpected outcome. In addition, given the unusual physico-chemical properties of NPs, perhaps more weight should be given to the pre-clinical data sets, and more time spent using the data to ponder the unexpected before deciding the dose and clinical care plan for the very first human to receive a new drug.

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Conclusions and remarks

Appropriate risk assessment in relation to health and safety of NPs used in medicine is essential, in order to ensure ethical, societal and regulatory acceptance, and public confidence. Many knowledge gaps concerning the health impact assessment of therapeutic NPs must be filled. There is presently no consensus on the most suitable toxicity tests, models for exposure assessment, and standardized testing strategies to evaluate possible hazards of therapeutic NPs for human health. Long-term effects of chronic exposure in humans of these NPs must be assessed. Tools for detecting NPs *in situ* must be developed for many of

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these therapeutic NPs. Bio-transformation of therapeutic NPs in the human body, their interaction with biological systems as well as adsorption, distribution, metabolism, transformation, degradation and excretion (ADME) in living systems should be studied in conjunction with a characterisation of NPs in terms of size distribution, surface properties, biopersistance, and stability of original and modified NPs in different biological media.

Acknowledgements

We acknowledge the support of the European Commission 7th Framework Programme for the NanoTEST (Health-2007-1.3-4, Contract no: 201335), NanoImpactNet (NMP4-CA-2008-218539, Grant Agreement 218539) and NANoREG (NMP.2012.1.3-3 NMP.2012.1.3-3, Grant Agreement no: 310584) projects..

Declaration of Interest

The authors declare to have no relevant affiliation or financial involvement with any organisation or entity with a financial interest or conflict concerning the information presented in this manuscript.

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List of Tables

Table 1: A few examples of therapeutic nanoparticles

Drug	**Polymer platform**	**Biological advantages**
Doxorubicin	PEG-liposomes chitosan, PLGA, etc	Decreased cardiotoxicity
5-fluorouracil	PEG-dendrimers, PEG-micelles	Increased half-life
Tamoxifen	PEG-nanospheres	Increased half-life and solubility
Cisplatin/carboplatin	Liposomes	Increased half-life
Methotrexate	PEG-copolymers, folic acid-PANAM	Decreased toxicity
Camptothecins	Liposomes	Increased solubility and stability

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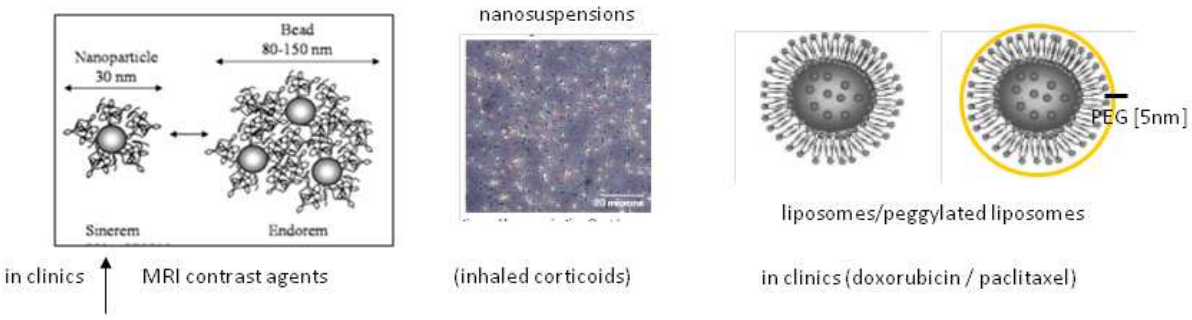

Figure 1. Examples of therapeutic NPs in clinics: MRI contrasts agents, mainly represented by iron oxide NPs; nanosuspensions of therapeutic drugs, for example inhaled corticoids; and PEG-gylated liposomal anti-cancer chemotherapeutics.

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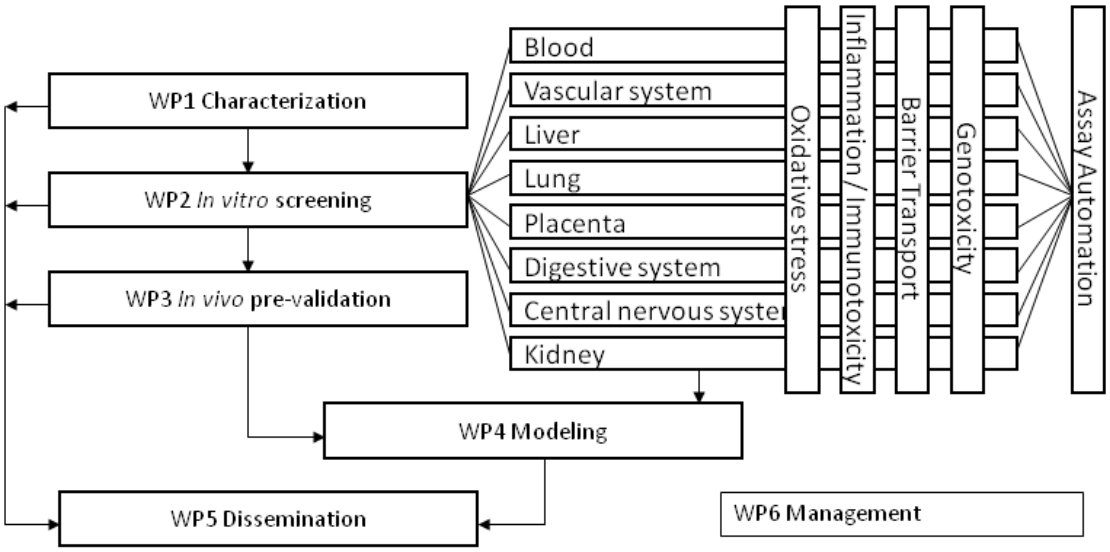

Figure 2. Work package structure of the NanoTEST project and structure of in vitro screening programme; cross-cutting topics cover all investigated organs

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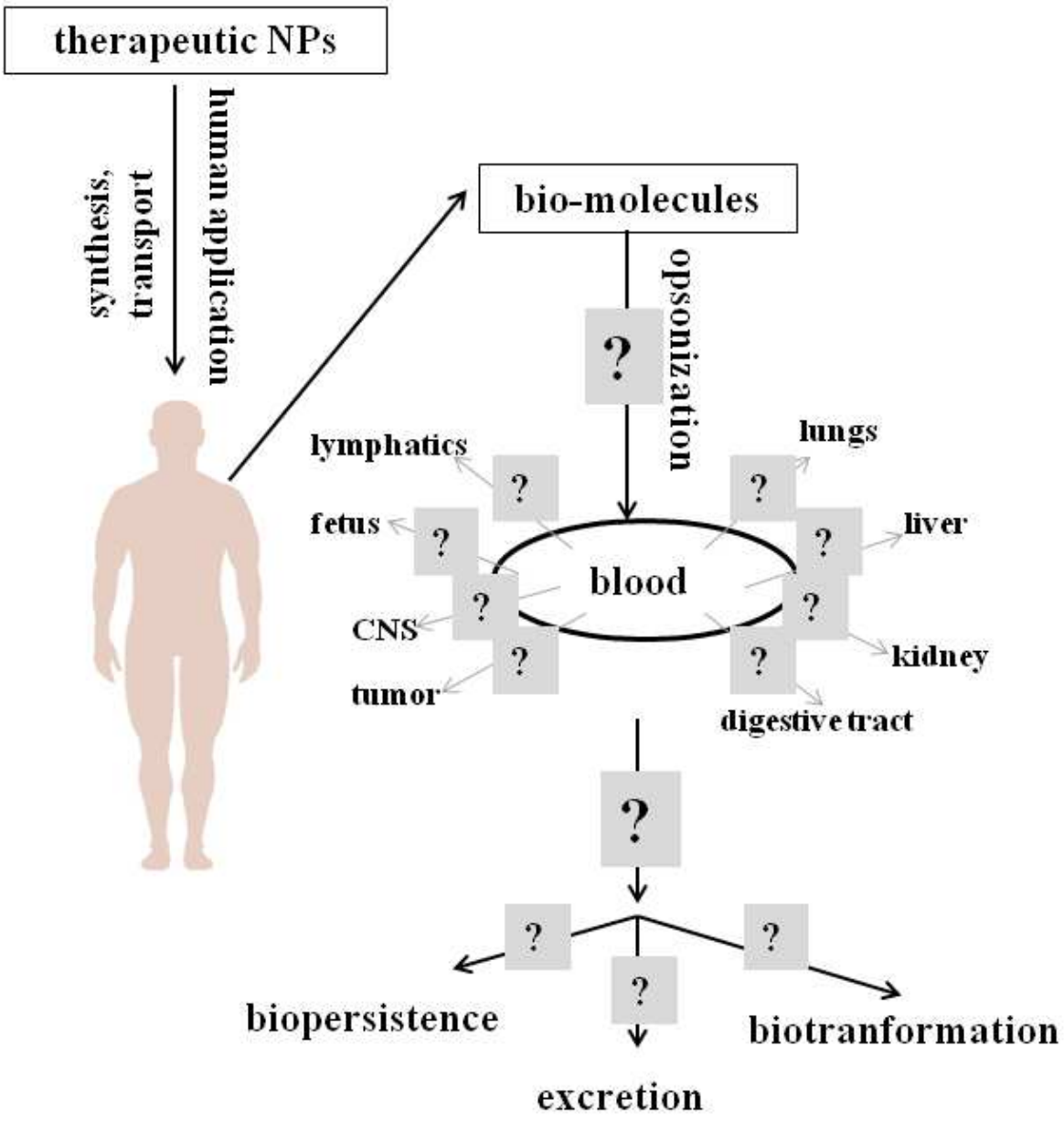

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Figure 3. Knowledge gaps in the life cycle of therapeutic NPs in humans

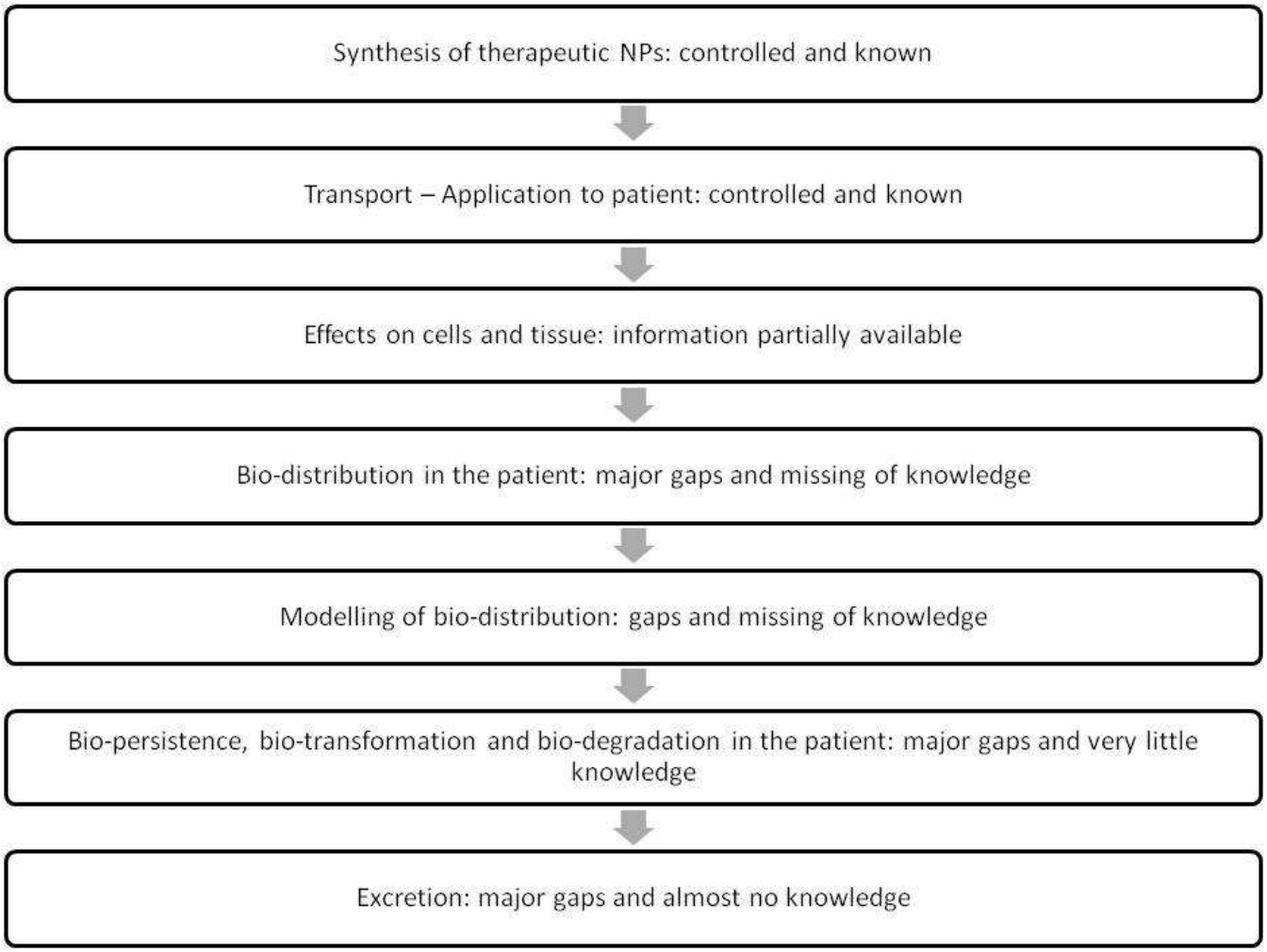

Figure 4. Overview of gaps in our knowledge for risk assessment of therapeutic NPs

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