A common European approach to the regulatory testing of nanomaterials

NANoREG

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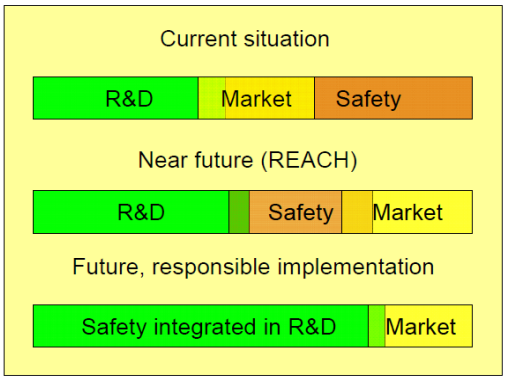
1 Description of task

1.1 Keeping pace with innovation

'Safe by design' is a concept that has been traditionally developed and applied in the engineering and construction sectors and which is now gathering momentum amongst scientists, regulators, and industry for use in the development of manufactured nanomaterials. The turn towards applying the concept of safe by design to the development of nanomaterials has been encouraged by some of the significant challenges facing regulation as a means of guaranteeing safety in this field. For example, a report by the UK Royal Commission on Environmental Pollution (2008) concluded that there is a fundamental problem with relying solely on regulatory instruments because there is often a considerable time lag between innovation, the products that result from it, and the subsequent case for amendment or development of regulations. This has also created a growing awareness amongst stakeholders that responsible innovation is required. That is, innovation that is directly oriented towards social and environmental needs, actively seeks to anticipate potential future impacts, carefully considers social and ethical issues and adapts innovation trajectories where necessary. In many countries, responsible innovation is supported by state-led initiatives, in a quest to ensure the safety, desirability and usefulness of emerging innovations for both society as a whole and the environment on which we depend. While regulation plays an ongoing important role for ensuring product safety, within the approach of responsible innovation, considerations of safety questions are also moved further up in the innovation chain. Here they become integrated into the research and development process itself, e.g. through the operationalization of concepts such as safe by design. Figure 1 contrasts the targeted transition from the current situation where regulation is the primary approach to guaranteeing safety and occurs post-market readiness of a product, to a new order where principles such as those of 'safe by design' are integrated into the development and manufacture of new nanomaterials, a key aim of NANoREG.

Figure 1. The changing role of safety in innovation through time



1.2 Beyond the State of the Art

Within NANoREG, WP6 seeks to explore possible routes of filling the increasing gap between innovation and risk analysis, by:

Being prepared: The development of more effective foresight of the potential impact of new manufactured nanomaterial (MNM) applications on human and environmental health by coupling horizon scanning to risk analysis. This will have strong potential to ensure the earliest identification of uncertainties and associated concerns for emerging innovations and will contribute to the better availability of safety data before marketing.

Safe by design: The safe by design concept aims at creating an integrated research strategy, which enables the consideration of safety aspects for humans and the environment throughout the product/material design phase. Such an approach maximizes resource use and expedites the development of products containing MNMs and new nanomaterials that are safer by design. Two building blocks will be addressed in this activity. One based on better exploiting existing knowledge and tools, and where possible applications of those tools already accepted for regulatory testing. The second building block will focus on how and with which toxicity information for the safe by design approach will best fit the requirements.

Turning risks into business opportunities: The requirements placed on industry to comply with future legislation might provide new business opportunities for standardisation and testing laboratories, as well as high tech industries who can translate the issues raised to investigate (eco)toxicity or exposure into efficient tools. Eventually the principles of safe by design will evolve into practical measures for product and material design that can also then offer new business opportunities.

With these aspects in mind and the ability to address them within NANoREG, the objective of creating a future sustainable market should be one step closer to what has been before.

1.3 Aim of deliverable

The safe by design concept has gained interest over recent years as it aims to reduce potential health and environmental risks at an early phase in the innovation process. In addition to its

development and use in construction and engineering sectors, this concept has also had a long history of successful deployment in the domain of drug development. In early phase drug development, new chemical entities are screened in parallel for both their efficacy and their potential for toxicity. High throughput testing in this type of research serves the question of whether benefit-risk ratios will likely to be positive. Such an approach might therefore include relevant building blocks for the uptake and development of the safe by design concept for MNMs. Turning this concept into effect will, however, be a significant challenge. It should be kept in mind that in drug development these types of tests have a kind of guiding function, leading to the most optimal functionality-efficacy-toxicity combination. However, full verification for drugs has to be proved by extensive in vivo testing in experimental animals and in humans. How such methods can be used for chemicals or MNMs used in consumer products will therefore be investigated in this deliverable, as well as how regulatory accepted approaches for drug development can be applied to the safe by design approach for MNMs. For this task, special attention needs to be paid to multidisciplinary collaboration between various types of expertise, such as (eco)toxicologists, in vitro specialists, and risk assessors who have experience with regulatory requirements for risk assessment of medical dossiers and for chemical substances (like REACH), in particular MNMs.

The aim of this deliverable (D6.3) is to evaluate which aspects of the drug development approach can contribute to the safe by design concepts for MNMs.

2 Description of work & main achievements

2.1 Introduction

The main goal of this deliverable is to compare the testing approach adopted in drug development to present MNMs safety testing and to evaluate which activities of the drug development approach can provide an added value for the safe by design concepts of MNMs.

In brief, the safe by design concept will be described, followed by the process of drug development (from discovery to market launch), MNM development (requirements for REACH) and finally the comparison between toxicity tests used for drug development and for MNM development will be made. The main questions which will be addressed in deliverable D6.3 are:

- What can we learn from the drug development process?
- Which aspects are useful for MNM development?
- What is an ideal process for safe by design?
- What are the critical questions in the drug development process?
- Do they also apply for the nanomaterials testing?
- Which toxicity tests are used in drug development?
- Are those tests applicable for MNMs?
- Which toxicity tests have been used for MNMs development until now?

Definition of a nanomaterial

A clear definition to discriminate nanomaterials from other materials is prerequisite to include provisions for nanomaterials in legislation. In October 2011, the European Commission published the 'Recommendation on the definition of a nanomaterial'. The European Commission based its recommended definition mainly on a reference report by the European Commission Joint Research Centre (JRC) (Lövestam et al., 2010) and a scientific opinion by the SCENIHR (SCENIHR, 2010). Inevitably, the final wording, and especially the thresholds, comprise political compromises as well. In its Recommendation (EU, 2011) the European Commission states that:

‘Nanomaterial’ means a natural, incidental 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 nm–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%.

Alternatively, it is stated that a material should be considered as falling under the definition where the specific surface area by volume of the material is greater than 60 m^2/cm^3. The Recommendation also includes definitions for ‘particle’, ‘agglomerate’ and ‘aggregate’. The Commission foresees a review of the definition by December 2014, particularly focusing on the appropriateness of the 50% limit.

The recommendation contains a rather broad description of the term nanomaterial. It potentially includes all kinds of nanomaterials/nanoparticles irrespective of their origin. The definition thus also covers natural nanoparticles, such as volcanic ashes, as well as incidental nanomaterials like nanoparticles originating from human activity such as exhaust of combustion processes, and the intentionally manufactured nanomaterials/nanoparticles by industry as they may be used in various industrial processes and consumer products. With the definition the Commission solely aims to identify substances within a specific size range and explicitly does not aim to classify nanomaterials as intrinsically hazardous. For this purpose it is appropriate to use a broad description, especially since no distinction can be made on the origin of the particle when only size is measured. However, as with other materials, distinctions between natural, incidental or manufactured materials need to be made in the specific areas of legislation since the need for such a distinction will be related to the purpose of that legislation.

The Commission states that the definition should be used as a reference for identifying nanomaterials for legislative and policy purposes in the European Union. However, whether defining a material as nanomaterial has regulatory consequences should be decided on in the specific regulatory frameworks. It is also indicated that for certain regulatory frameworks deviations may in some cases be necessary, either to exclude materials that fall within the definition or to include materials that are beyond the definition. For this reason, the option is given by the Commission to adjust the number size distribution threshold to a value between 1% and 50%.

Furthermore, recent documents published under the JRC Science and Policy report series have published two reports, and planning a third one, where they have collected information on scientific-technical issues that should be considered when reviewing the current EC nanomaterial definition. The first report discusses how the different nanomaterials definitions are used and how they diverge. The second report also puts the EC nanomaterial definition in perspective by

comparing it with other existing nanomaterial definitions, thereby identifying the most prominent characteristics of the EC definition.

Based on the feedback received regarding the current definition, compiled in the first report of the series, and its assessment, presented in the second report, the JRC is now working on a set of indications on how the definition could be modified to improve its clarity, effectiveness and implementability. These recommendations will be included in a final report (Part 3 of the series), which is expected to be released in 2014.

Within NANoREG, the definition recommended by the Commission will be used for identifying nanomaterials.

2.2 Safe by Design concept

2.2.1 Introduction

Safety by design or safe by design is originally a concept that was developed and utilized by engineers, particularly those working within the construction industry. The basic idea is that in the design and development of products, it is important to consider and incorporate safety considerations. The concept has also been used in the field of drug development and it has also been taken up, adapted and expanded within other fields of industry, such as in plant design, healthcare, and social security and crime prevention. The specific terms used to describe the concept may vary slightly between different fields of industry. Engineers, plant developers and the construction industry usually refer to the terms ‘Safety by design’ or ‘Safety in design’, while the terms ‘Safe by design’ or ‘Safer by design’ are more often used in healthcare and city planning. However, these terms closely resemble each other and are sometimes used interchangeably; for example, a quick literature search reveals that within the field of nanotechnology, authors have used ‘Safety by design’, ‘Safe by design’, and ‘Safer by design’ without distinction. Within NANoREG, we have chosen to use the term ‘Safe by design’.

The concept has also recently been adapted within other fields, such as can be seen in the development of ‘Green chemistry’ and ‘Inherent safety’ (see text box). The desire to anticipate potential impacts of a product and include consideration of these into product design and development is also an idea captured within the concept of lifecycle analysis, although this has traditionally been more oriented towards broader sustainability considerations (and particularly energy use) rather than human health and environmental safety. The emerging notion of responsible innovation also shares certain characteristics with that of safety by design, namely that of considering potential societal impacts of a product at an early stage in the innovation process. However, responsible innovation as a concept has a broader frame than that of safety by design, taking into account not only questions concerning human health and environmental safety, but also social and ethical dimensions and the specific question of social need and utility.

Text box 1. Inherent Safety

An early advocate of the idea of paying attention to safety issues in the beginning rather than at the end of a production process was Trevor Kletz, who published his paper "*What you don't have, can't leak*" back in 1978. Kletz further developed his ideas into the concept of 'Inherent Safety': designing processes that have an intrinsically low level of hazard, instead of trying to control hazards by protective systems. Inherent Safety is mainly applied in the chemical and production/engineering industry. It comprises four main principles:

1. *Minimize*, or Intensify: reducing the quantity of material or energy contained in a manufacturing process or plant, and performing a hazardous procedure as few times as possible.
2. *Substitute*: replace hazardous material or processes with less hazardous alternatives.
3. *Moderate*, or Attenuate: using hazardous materials in their least hazardous forms, and use less severe process conditions (e.g., perform chemical reactions at a lower temperature).
4. *Simplify*: design processes, processing equipment and procedures to be as robust as possible, instead of using excessive protective layers.

Text box 2. Green chemistry

The concept of 'Green chemistry' originates from the field of chemistry and is defined as "*the design of chemical products and processes to reduce or eliminate the use and generation of hazardous substances"*. The twelve principles of green chemistry were developed by Paul Anastas and John C. Warner in 1998 and are presented below:

1. *Prevent waste*: Design chemical syntheses to prevent waste. Leave no waste to treat or clean up.
2. *Maximize atom economy*: Design syntheses so that the final product contains the maximum proportion of the starting materials. Waste few or no atoms.
3. *Design less hazardous chemical syntheses*: Design syntheses to use and generate substances with little or no toxicity to either humans or the environment.
4. *Design safer chemicals and products*: Design chemical products that are fully effective yet have little or no toxicity.
5. *Use safer solvents and reaction conditions*: Avoid using solvents, separation agents, or other auxiliary chemicals. If you must use these chemicals, use safer ones.
6. *Increase energy efficiency*: Run chemical reactions at room temperature and pressure whenever possible.
7. *Use renewable feedstocks*: Use starting materials (also known as feedstocks) that are renewable rather than depletable. The source of renewable feedstocks is often agricultural products or the wastes of other processes; the source of depletable feedstocks is often fossil fuels (petroleum, natural gas, or coal) or mining operations.
8. *Avoid chemical derivatives*: Avoid using blocking or protecting groups or any temporary modifications if possible. Derivatives use additional reagents and generate waste.
9. *Use catalysts, not stoichiometric reagents*: Minimize waste by using catalytic reactions. Catalysts are effective in small amounts and can carry out a single reaction many times. They are preferable to stoichiometric reagents, which are used in excess and carry out a reaction only once.
10. *Design chemicals and products to degrade after use*: Design chemical products to break down to innocuous substances after use so that they do not accumulate in the environment.
11. *Analyze in real time to prevent pollution*: Include in-process, real-time monitoring and control during syntheses to minimize or eliminate the formation of byproducts.
12. *Minimize the potential for accidents*: Design chemicals and their physical forms (solid, liquid, or gas) to minimize the potential for chemical accidents including explosions, fires, and releases to the environment.

2.2.2 **What is Safe by Design**?

Sustainable innovation or responsible implementation of a new technology are approaches which demand attention to incorporating the questions about safety for human beings and the environment at an early stage of the innovation chain. This already happens in the regular process of product development to some extent, but especially in the instance of new technologies, it is not clear whether regulation provides a comprehensive answer to the question of whether a product is safe. There are various initiatives to achieve a change in this. One example of this is REACH, to obtain relevant information on safety for human beings and the environment for chemical substances which are currently on the market or are yet to be launched. The suitability of REACH for nanomaterials is still a matter of discussion between and within various stakeholder groups. Within the Safe by Design concept, the functionality of a material and its toxicity are considered in an integrated way. Safe by Design has traditionally been about incorporating safety considerations into the design, construction and maintenance of engineered products and workplaces. It can be formally understood as the integration of hazard identification and risk assessment methods early in the design process to eliminate or minimise the risks of injury throughout the life of a product or structure being designed, including construction, use, maintenance and destruction. It encompasses all design factors including facilities, hardware, systems, equipment, products, tooling, materials, energy, controls, layout and configuration. Safe by design can also be addressed as 'Prevention by Design' with the goal of designing out occupational hazards by focusing on hazard elimination and substitution (NIOSH, 2014). Safe by Design in this WP is about incorporating considerations of potential health and environmental safety concerns into the research and development phase of an innovation process and where necessary adapting the process and/or product design so as to create safer outcomes.

A safe design approach begins in the conceptual and planning phases with an emphasis on making choices about design for the materials used, the methods of manufacture or construction, the potential uses, and the routes for disposal to enhance the public and environmental safety of the finished product. The designer therefore needs to consider how safety can best be achieved across each of the lifecycle phases. A number of countries already include Safe by Design requirements in their health and safety legislation for certain sectors. This is, for example, to ensure that hazards and risks that may exist in the design of a workplace are eliminated or controlled at the design stage as far as reasonably practicable. Regardless of the legal environment, it should be recognized that most innovators aim to produce designs that are safe and without risks as far as reasonably practicable, however direct support of, access to and integration of safety science and risk-based research may not always be facilitated.

2.2.3 **What are the benefits of Safe by Design?**

The benefits of a Safe by Design approach includes prevention of injury and disease to those constructing, using or maintaining the product/structure, prevention of potential harm to humans or elements of the environment both during the manufacturing process and exposure to the product,

improved usability and public acceptability of products, systems and facilities, improved productivity and resource efficiency, reduced costs (especially the health and environmental costs that are often externalized), better prediction and management of production and operational costs over the lifecycle of a product and ease in meeting compliance with legislation.

Although the benefits of the Safe by Design concept are clear, the practical interpretation of the concept is not. For example, which data are needed at exactly which stage of the innovation process? Who should generate these data? Who will decide whether or not to move on with the innovation, and how? What kind of tool(s) could support this decision process? Could (elements of) 'Inherent safety' and 'Green chemistry' be adopted into 'Safe by design' for nanomaterials? With this deliverable we aim to make a start with the practical interpretation of safe by design for MNMs.

2.2.4 **Safe by Design for nanotechnology**

Nanotechnology deals with materials on the scale of nanometres (1 nanometre equals 1 millionth of a millimetre) and enables the handling of material on a molecular level, allowing alteration of the properties of this material. The nanoparticles thus created exhibit different characteristics than larger particles, which alters their behaviour. In this way, a new generation of technological applications is formed, opening up new possibilities in a wide range of fields, varying from health care and food to the environment and agriculture. Nanoparticles have been incorporated in many different types of products, and the novel properties of nanomaterials offer great promise to provide new technological breakthroughs. However, nanotechnology is an emerging technology that may pose potential health and safety risks throughout its product life cycle. The health risk of a nanoparticle is a function of both its hazard to human health and its exposure potential. It is prudent for companies to try to mitigate the potential risks of nanoparticles during the design stage rather than downstream during manufacturing or customer use.

In 2010, Morose proposed five design principles for product designers to use during the design stage for products that contain nanoparticles. By using these design principles, the health risk of the nanoparticle may be mitigated by potentially lowering the hazard and/or the exposure potential of the nanoparticle. Morose specified the 'Safe by design' concept into a design strategy specifically for nanomaterials: "*Design for Safer Nanotechnology*", which consists of the following 5 principles (Morose, 2010):

1. **S** for Size, Surface and Structure: these parameters affect the fundamental properties of the nanomaterial, such as melting point, conductivity and reactivity. Size, Surface and Structure could be modified to decrease the hazard and exposure potential while maintaining functionality.
2. **A** for Alternative Materials: replacing hazardous materials with less hazardous materials. Changes in functionality or costs should however be considered.

3. **F** for Functionalization: adding certain atoms or molecules to the nanomaterial to change its properties, for example increase the solubility by adding adducts. In contrast to Encapsulation, Functionalization does not completely cover the nanomaterial surface.
4. **E** for Encapsulation: coating of the nanomaterial.
5. **R** for Reduce the quantity: in case none of the above measures can be applied without losing important desired product features, the hazardous nanomaterial still has to be used, but the quantity should be minimized.

The twelve principles of Green chemistry (see Text box 2, page 12) show that this approach does not solely addresses EHS issues of the final product; the approach also incorporates EHS aspects of the manufacturing process itself. The Green chemistry approach is therefore considered appropriate for use in nanotechnology to overcome the current focus on nano-products. Furthermore, the Green chemistry approach has been successfully applied in the preparation of highly functionalized products, for example pharmaceuticals. These diverse chemical substances have a strong correlation with the heterogeneous nature of nanomaterial containing products. Overall, the Green chemistry method seems to be well suited to address EHS aspects in nanotechnology (Jacobs et al., 2010).Jacobs and colleagues followed a different but similar approach as Morose, starting from the concept of Green chemistry. From the 12 principles of Green chemistry they deducted four general concepts for '*Green nanotechnology'*:

1. **Product safety**: designing products with an as low as possible hazard potential while maintaining their desired function. This relates to the Green chemistry principle number 4.
2. **Low environmental impact**: design products that can be reused, recycled, or broken down in the environment. This concept integrates the Green chemistry principles number 7 and 10.
3. **Material and energy efficiency**: maximizing the incorporation of recycling of materials, thus preventing waste, and minimizing the use of energy. This concept integrates the Green chemistry principles number 1, 2, 6, 8, 9, and 11.
4. **Process safety**: design an as safe as possible manufacturing process. This concept integrates the Green chemistry principles number 3, 5, 11, and 12.

Movia and colleagues applied the safe by design approach to optimize the development of biocompatible nanomaterials for therapeutic applications. There the efforts are focused on characterizing the physical, chemical and biological properties of the core material, followed by "layering" as a method to produce safe nano-enabled theranostics within a personalised medicine approach (Movia et al., 2013). Table 1 gives an overview of the different concepts and their applicability to nanotechnology.

Table 1. Overview of terms related to 'Safety by design' and their possible application in nanotechnology

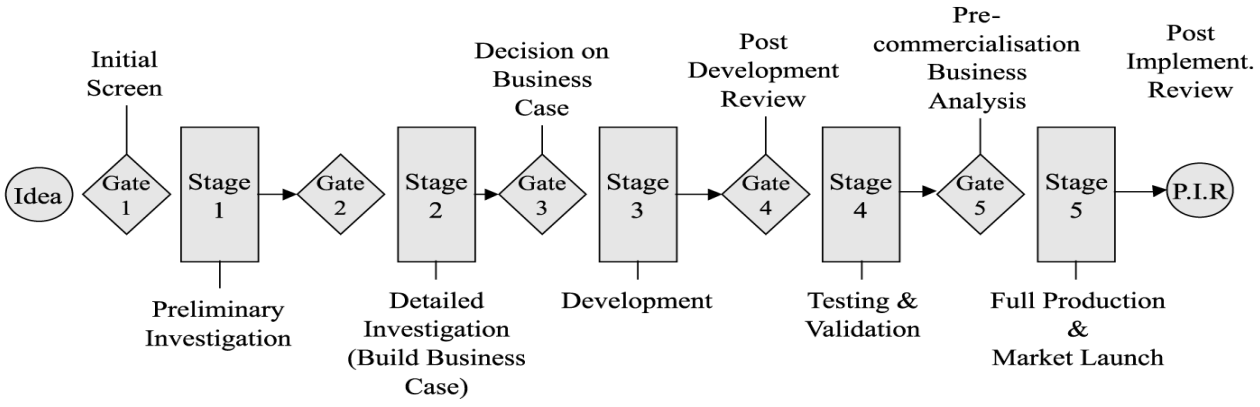
Term	Field of origin	Description	Applicability to Nanotechnology	References
Inherent safety	Chemical industry (Production)	1.Minimize 2.Substitute 3.Moderatei 4. Simplify	Especially the principles Minimize: minimizing exposure to MNMs; Moderation: nanoparticle properties could be designed to engineer less hazardous particles, e.g. by modifying the size, surface and structure, coating, or adducts; and Substitution: less hazardous forms of nanomaterials could be used.	Jacobs et al., 2010 Amyotte, 2011
Green chemistry	Chemical industry	1.Prevent waste 2.Maximize atom economy 3.Design less hazardous chemical syntheses 4.Design safer chemicals and products 5.Use safer solvents and reaction conditions 6.Increase energy efficiency 7.Use renewable feedstocks 8.Avoid chemical derivatives 9.Use catalysts not stoichiometric reagents 10.Design chemicals and products to degrade after use 11.Analyze in real-time to prevent pollution 12.Minimize the potential for accidents	Can be applied to nanotechnology, however, for hazard reduction of nanomaterials it is not sufficient as specific physical parameters may also play a role. The focus on the entire production chain, including energy flows and waste management, is recommendable to be adopted in the Safe by design concept.	Jacobs et al., 2010 Anastas and Eghbali, 2010
Design for Safer Nanotechnology	Nanotechnology	1.Size, surface and structure 2.Alternative materials 3.Functionalization 4.Encapsulation 5.Reduce the quantity	Developed for nanotechnology with the focus on decreasing the hazard potential of the nanomaterial.	Morose, 2010
Green Nanotechnology	Nanotechnology	1.Product safety 2.Low environmental impact 3.Material & energy efficiency 4.Process safety	Overlaps with 'Inherent safety' and 'Design for Safer Nanotechnology', but adds a focus on process efficiency in terms of energy and waste reduction.	Jacobs et al., 2010

2.2.5 **What is our approach to Safe by Design?**

Our approach of the Safe by Design concept is to develop new products where functionality and safety are tested in an integrated way through the development process phase. This integrated approach demands multi-disciplinary collaboration, knowledge and resources. Moreover, during the development of this approach several aspects have to be addressed, for example i) the motivation for different parties to participate, ii) the role of trust, and iii) whether this can be a self-regulating system or whether leadership or responsibility is expected from a stakeholder. These process-oriented sides deserve more attention in Safe by Design projects, because it is a prerequisite for Safe by Design structurally being viewed as a concept rather than a project.

An innovation process is desirable to define and structure a Safe by Design approach. A widely implemented product innovation process model is the Stage-Gate idea-to-launch model (see Figure 2). The Stage-Gate product innovation system is a conceptual and operational map for moving new product projects from idea to launch and beyond, a blueprint for managing the new product development process to improve effectiveness and efficiency (Cooper, 2008).

Figure 2. Stage-Gate product innovation process



The Stage-Gate model was adapted by integrating the management of technology development (Technology Readiness Levels (TRLs)), which has led to a new innovation model (Figure 3). The integration of TRLs into the Stage-Gate model has led to an innovation process, which consists of five stages:

1. Basic research: Initial scientific research has been conducted, principles are qualitatively postulated and observed and research ideas and protocols are developed.

2. Research feasibility: Applied research advances and early stage development begins. Studies and laboratory measurements validate analytical predictions of separate elements of the technology.

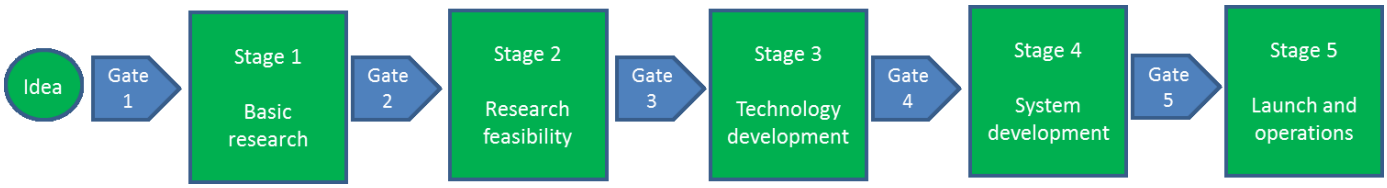
3. Technology development: Design, development and lab testing of components/processes. Results provide evidence that performance targets may be attainable based on projected or modeled systems.

4. System development: System Component and/or process validation is achieved in a relevant environment.

5. Launch and operations: Actual system proven through successful operations in operating environment, and ready for full commercial deployment.

This model can give guidance to develop the Safe by Design concept within NANoREG. The structure of this innovation process will be used in this deliverable to make an overview of the toxicity test used for drug development and for MNM development.

Figure 3. New innovation model



Within WP 6 of NANoREG, our approach to the concept of safe by design is:

a) for all stakeholders to contribute to a process in which public health and environmental safety should be considered at all stages of the innovation process

b) for there to be a good interaction between risk-based research and product development (innovation)

c) for innovation processes to be able to specifically adapt design factors to take safety aspects into account

2.2.6 Who has responsibilities in relation to Safe by Design?

According to our approach to the concept, it is not only product innovators that have a responsibility to ensure their products are safe by design. There are various stakeholders that will need to meet responsibilities if the concept is to be successfully operationalized for MNMs. For example, risk-based researchers will have a responsibility to ensure that their work is accessible for and available to product innovators. Product innovators and governments will have a responsibility to ensure that the necessary funding to support the

required safety research is available. International standards bodies will have a responsibility to develop appropriate standards for safety testing that can be applied across certain sectors and for specific applications. All actors along an innovation chain will have responsibility to ensure transparency concerning the presence and role of different MNMs in their products and processes. Media organisations may also have responsibility to accurately report on the potential risks and how they may have been accounted for in the (re)design of different products. In addition, insurers and consumers may also have responsibilities supporting the safe by design concept.

2.2.7 Does Safe by Design lead to risk elimination?

It does not seem reasonable to expect that all risks will be eliminated in a safe by design approach. By applying such an approach, a de-risking step is taken to decrease the level of certain uncertainties of risks and where possible, hazards should be avoided. However, there will be many situations and cases where it is either impossible or necessarily desirable to design out all hazards. Where hazards cannot be avoided, innovators should reduce the risks associated (re-risk) with the hazard, e.g. by minimizing exposure scenarios. Arrangements can be made to avoid foreseeable risk in preparing a design eliminating hazards giving rise to the risk and/or reducing exposure to any remaining hazards.

2.3 Drug development

Drug discovery and development is an expensive process due to the high costs of R&D and human clinical tests. The average total cost per drug development varies from € 1 to 2.3 billion with a typical development time of between 10-15 years. Therefore, the development of new formulations has to be based around efficiency and operational excellence within the drug discovery and development processes to deliver innovative solutions to patients.

New drug R&D involves the identification of a target (usually a protein) and the discovery of some suitable drug candidates (usually chemical compounds) that can block or activate the identified target. After an initial lead candidate verification and validation (called titration process), clinical testing, as the most extensive and expensive phase in drug development, is carried out in order to obtain the necessary governmental approvals. In Europe, drugs must be approved by the European Medicine Agency (EMA); whereas in the US, drugs must be approved by the Food and Drug Administration (FDA).

Regarding the drug development aided with nanotechnology (called nanomedicine), EMA has established an Agency's Committee for Medicinal Products for Human Use (CHMP) (ad hoc expert group on nanomedicines established since 2009), which have already led to the approval of a number of medicines based on nanotechnology.

2.3.1 **Drug development process**

The process of drug development is generally divided into two stages: new lead discovery (preclinical research) and new product development (clinical development). MNM falls under the first stages under most of the cases unless there is a previous similar case to be used for comparison. The time-lines and expenses of these stages are radically different. This section will attempt to provide a broad overview of the activities required to successfully complete each phase of the development pathway.

In order to market a drug, pharmaceutical companies are required to prove a clear risk to benefit ratio which can be captured under the following three aspects:

1. the drug is safe to be used with patients (end-users);
2. it is effective in treating the specific disease for which it was designed despite overall toxic side-effects to the end-user; however the benefit outweighs the risks associated; and;
3. the drug can be manufactured cleanly and reproducibly each time that it is prepared.

Text box 3. Safety, efficacy and effectiveness

Safety is often measured by toxicity testing to determine the highest tolerable dose or the optimal dose of a drug needed to achieve the desired benefit. Studies that look at safety also seek to identify any potential adverse effects that may result from exposure to the drug. Efficacy refers to whether a drug demonstrates a health benefit over a placebo or other intervention when tested in an ideal situation, such as a tightly controlled clinical trial. Effectiveness describes how the drug works in a real-world situation. Effectiveness is often lower than efficacy because of interactions with other medications or health conditions of the patient, sufficient dose or duration of use not prescribed by the physician or followed by the patient, or use for an off-label condition that had not been tested.

This process begins with the Discovery Research where new compounds are found and assuring that they are safe enough to be used in humans (this is often linked to phase 1 clinical trial; however recent development also introduced phase 0 trials where sub-groups of patients can consent to the drug testing). The tasks required to achieve this goal are numerous and require the expertise of a number of departments. These include:

- Research Planning - the management team, which sets therapeutic targets, budgets, and resources.
- Chemistry - whose responsibilities are to prepare new chemical entities (NCEs), which can be screened for biological activity and to prepare compounds which have been found to be active (new leads) in quantities sufficient for advanced testing.

- Pharmacology/Molecular Biology/Screening - examines each NCE in a set of high throughput screens. This is referred to as primary screening, which is used to determine if the compound is active (e.g. does it bind to the test enzyme more tightly than the control compound) or inactive. The majority of compounds tested do not show activity. However, when a molecule demonstrates high affinity for a target receptor, more rigorous testing (secondary screening) follows to assure that the initial results were correct.

- Safety Evaluation - demonstrates that the NCE and its metabolites do not accumulate and do not cause harm during short-term administration. These tests are generally carried out in bacteria and yeast (genetic effects) and at least two animal species.

- Formulations Research - develop a dosage form (pill, tablet, capsule, etc.) that is absorbed into the blood stream when administered and is stable when stored for long periods of time. The concentration in the blood is an important factor in early development. The potential new drug must reach and maintain a level sufficient to sustain its biological effect. These studies are initially conducted in animals, however, doses for human studies will be derived from these studies.

- Process Research - manufactures the NCE in quantity for advanced testing, dosage form development, and other support activities.

- Legal Affairs - writes and files the patents necessary to protect a company's inventions. Patents must be filed before any public disclosure of a new compound's structure or activity. If the compound is reported (disclosed) before the patent is filed, it is considered to be public property.

- Research Administration - collects the material generated by all of the departments and formats it into a request for exemption so that the NCE can be tested in humans. This submission is the Investigational New Drug application or IND.

The above is a very brief overview of preclinical research. This process begins with an analysis of the market potential for a given therapy and ends with three documents which detail the efforts of a number of professionals - a patent, a statement of clinical attractiveness, and an IND. These documents initiate the next phase in the drug development process - clinical trials.

Once the company receives approval to study an NCE in humans, several parallel events take place, which are organized in phases. While there is general agreement about what constitutes each phase, there is no standard definition. The clinical studies section is organized as follows:

- Phase 0: Phase 0 clinical trials, developed in response to the FDA's recent exploratory Investigational New Drug (IND) guidance, are intended to expedite the clinical evaluation of new molecular entities. The exploratory IND supports the performance of first-in-human testing of new investigational agents at subtherapeutic doses based on reduced manufacturing and toxicologic requirements, allowing the demonstration of drug-target effects and assessment of pharmacokinetic-

pharmacodynamic relationships in humans earlier in clinical development (Kummar, 2008).

- Phase I - Establishes safety in humans. The patient population is a limited (20 - 40) group of healthy volunteers. The studies are used to determine toxicity, dosages (formulations and amounts), blood levels, excretion profiles, and pharmacokinetic profiles.
- Phase II - Establishes that the NCE is effective in treating the disease in limited patient populations (2A, about 100 subjects) and medium populations (2B, about 300 subjects). Phase II is generally when adverse effects of a potential drug are observed. The studies are used to determine toxicity, compatibility with other medications, bioavailability/bioequivalence of different formulations and a variety of other effects.
- Phase III - During this phase, a variety of patients with varying degrees of the disease are studied. Multicentre, controlled trials on thousands of patients are run to complete the establishment of safety, efficacy and dosage for the compound.
- Phase IV - Post marketing surveillance is used to monitor the drugs efficiency in treating large populations, locate any reports of adverse effects, and assess the relative efficacy of the drug. All reports about a drug, which appear in the public are maintained by the company marketing the drug.

In addition to the clinical trials, development of a new drug also requires a number of supporting activities:

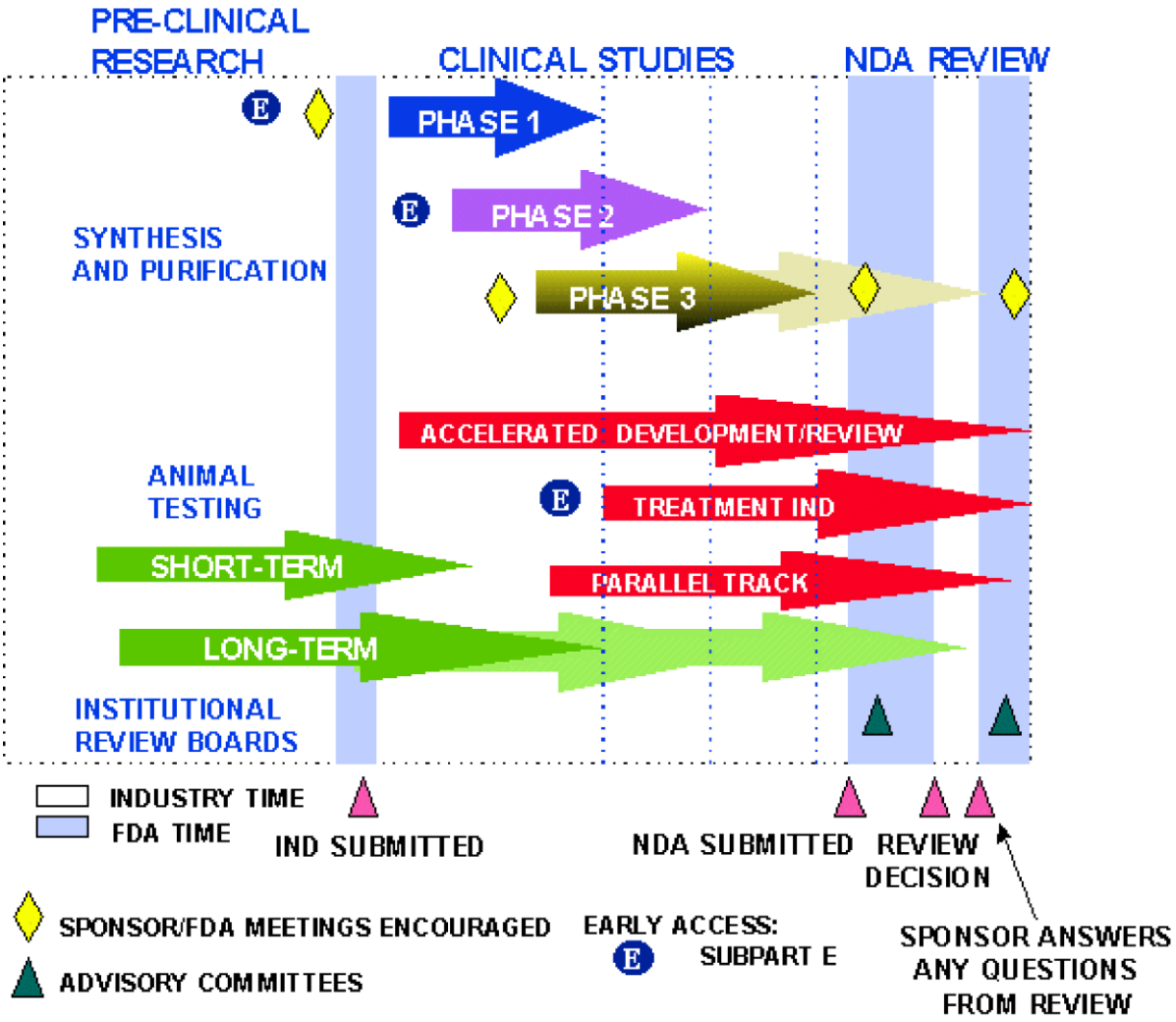
- Completion of manufacturing protocols
- Toxicology studies in animals to assure long-term safety
- Dosage formulation and stability
- Metabolism studies to assure that the drug does not accumulate or is not converted into a toxic substance

When all of these activities have been completed, the company prepares an NDA submission. Data from all of the clinical studies and the manufacturing efforts is collected and formatted into the appropriate reports. This collection of documents (which can occupy a large moving van), includes not only the interim and final study reports for all human and animal studies, but also all of the "raw data" that was used to produce the reports and details of the manufacturing processes that will be used to make the final drug.

There are several stages to drug development, each of which requires a significant investment of time, money, and resources. The overall drug development process is shown in Figure 4. Once the activity of the new chemical lead is confirmed, several parallel activities are initiated. Process research begins to develop new synthesis routes, which will permit the preparation of large quantities of the compound. At the same time, the safety evaluation

group starts a short term examination to determine the toxicity of the new compound. As laboratory results from these activities unfold, research scientists and clinicians begin developing plans for human trials, product packaging, and benefits for future studies. If all of these activities are successful, each of the studies, reports, and procedures are combined and formatted into the Investigational New Drug Application. While the research department may work to develop additional compounds within the same class (analogues) to serve as a backup to the primary clinical candidate, approval of the IND formally ends the research department's connection with the compound.

Figure 4. The New Drug Development Process: Steps from Test Tube to New Drug Application Review (CDER Handbook, 1998)



2.3.2 **Drug indentification**

One of the most successful ways to find promising drug candidates is to investigate how the target protein interacts with randomly chosen compounds, which are usually a part of compound libraries. This testing is called High Throughput Screening (HTS). Compound libraries are commercially available in sizes of up to several millions of compounds. The most promising compounds obtained from the screening are called *hits* – these are the compounds that show binding activity towards the target. Throughout a systematic HTS campaign, some of the hits are then promoted to lead compounds, which are further refined and modified in order to achieve more favourable interactions and less side-effects.

In recent years, in order to reduce the lead-compounds identification, expedite the titration process and gain valuable commercial and exploitable years, in silico modelling and virtual screening have gained a lot of momentum in the initial drug discovery desk assessment phase. Several advantages and disadvantages can be highlighted such as: 1) low costs, 2) no compounds have to be purchased externally or synthesized; 3) HTS testing can be refined to reduce the initial number of compounds to test experimentally, and 4) a large number of synthetic chemicals can be simulated that have not been synthetized yet. The main disadvantage is that it cannot substitute the real screening, such as HTS, which can experimentally test the activity of hundreds of thousands of compounds against the target.

Text box 4. High-Throughput Screening and in silico Screening

High-Throughput Screening (HTS) has become a standard method for drug discovery in the pharmaceutical industry. It is a process of screening and assaying a large number of biological modulators and effectors against selected and specific targets. HTS assays and techniques and are used for screening of different types of libraries, including combinatorial chemistry, genomics, protein, and peptide libraries (Szymański et al., 2012). The main goal of the HTS technique is to accelerate drug discovery by screening large compound libraries at a rate that may exceed a few thousand compounds per day or per week. It is of vital importance, because parallel and combinatorial chemical and biochemical synthesis generates a vast number of novel compounds. High-throughput screening methods are also used to characterize metabolic, pharmacokinetic and toxicological data about new drugs. HTS consist of several steps such as target identification, assay development, experimental screening and high-throughput library screening. The successfully tested and selected drugs called as leads, which undergo further screening process by testing in different phases of pre-clinical and clinical studies and then go for regulatory approval (Kramer et al). Among the many techniques used in HTS it is on importance to mention High-Content screening as the only quantitative technique, which has been extensively applied for assessing biological, drugs, nanomedicines and MNMs for safe applications and test the safe-by-design concept (Movia et al., 2013, Byrne et al., 2009). High-Content Screening (HCS) is also a method that is used in biological research and drug discovery to identify substances such as small molecules, peptides, or interference RNA that alter the phenotype of a cell in a desired manner.

Another method of lead identification is ‘virtual screening’ (also named in silico screening) which is defined as the ‘selection of compounds by evaluating their desirability in a computational model’. Compounds testing positive in screening have their potency and selectivity confirmed by in vitro biochemical or cellular assays. This is typically followed by functional biochemical and pharmacological testing in vitro, followed by pharmacodynamics and pharmacokinetic testing in vitro and in vivo (Tamimi et al., 2009).

In silico modelling as computational methods can be used to predict or simulate how a particular compound interacts with a given protein target. They can be used to assist in building hypotheses about desirable chemical properties (for example: solubility, protein binding in plasma (to enhance the drug half-life), partition coefficients, etc.) when designing the drug and, moreover, they can be used to refine and modify drug candidates. On this, three computational methods are used in the modern drug discovery process: Molecular Docking, Quantitative Structure-Activity Relationships (QSAR) and Pharmacophore mapping. Briefly, Molecular docking programs predict how a drug candidate binds to a protein target. QSAR formalizes what is experimentally known about how a given protein interacts with some tested compounds. QSAR models are used for *in silico* screening of compounds to investigate their appropriate drug candidate descriptors for the target. Where QSAR focused on a set of descriptors and chemical properties, Pharmacophore mapping is a geometrical approach, a combination of steric and electronic matching between the protein binding pocket and the ligand. A pharmacophore can be thought of as a 3D model of characteristic features of the binding site of the investigated targeted protein.

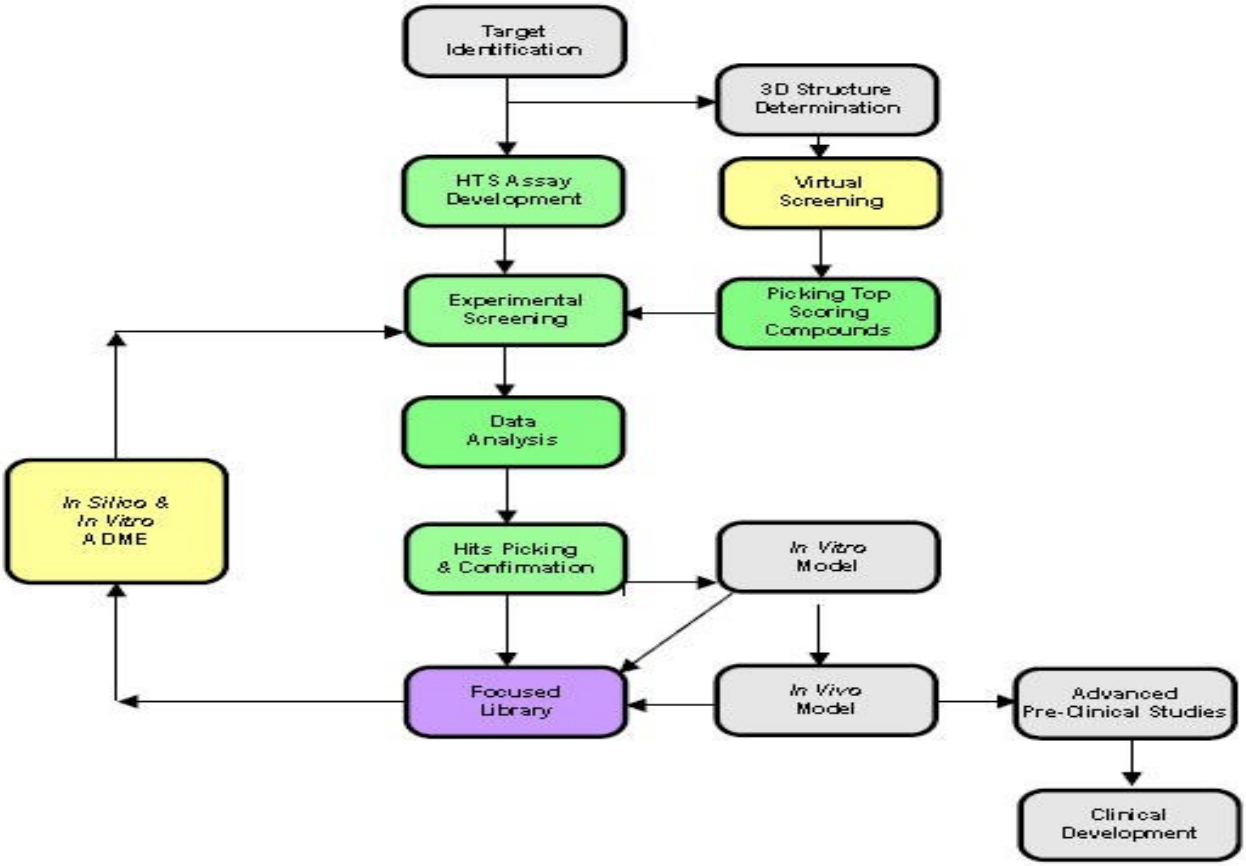
After the initial lead-compound identification the next step in transforming a molecule into a drug is evaluation in pre-clinical (pre-human) testing, which establishes the effectiveness and safety of the molecule in systems, which mimic those present in humans:

- Phase 1 - Perform initial human testing in a small group of healthy volunteers.
- Phase 2 - Test in a small group of patients.
- Phase 3 - Test in a large group of patients to show safety and efficacy.

If a drug passes through these various stages with good results of efficacy and safety, it is submitted to the appropriate government agencies for review.

- Phase 4 (post-market) – After a product has been approved, more studies are conducted to provide additional information about safety and effectiveness.

Figure 5. Schematic approach of drug identification process (from http://www.cityofhope.org/high-throughput-screening)



2.3.3 **Toxicity tests in drug development**

Preclinical testing analyses the bioactivity, safety, and efficacy of the drug by investigating the principal aspects associated with the physiology of the end-user, its administration route and the targeting principal. This testing is critical to a drug's eventual success and, as such, is scrutinized by many regulatory entities. During the preclinical stage of the development process, plans for clinical trials and an Investigative New Drug (IND) application are prepared. Studies taking place during the preclinical stage should be designed to support the clinical studies that will follow. Thus, the main stages of preclinical toxicology testing are grouped in the following:

- *Acute Studies*

Acute toxicity studies look at the effects of one or more doses administered over a period of up to 24 hours. The goal is to determine toxic dose levels and observe clinical indications of toxicity. Usually, at least two mammalian species are tested. Data from acute toxicity studies helps determine doses for repeated dose studies in animals and Phase I studies in humans.

- *Repeated Dose Studies*

Depending on the duration of the studies, repeated dose studies may be referred to as subacute, subchronic, or chronic. The specific duration should anticipate the length of the clinical trial that will be conducted on the new drug. Again, two species are typically required.

- *Genetic Toxicity Studies*

These studies assess the likelihood that the drug compound is mutagenic or carcinogenic. Procedures such as the Ames test (conducted in bacteria) detect genetic changes. DNA damage is assessed in tests using mammalian cells such as the Mouse Micronucleus Test. The Chromosomal Aberration Test and similar procedures detect damage at the chromosomal level.

- *Reproductive Toxicity Studies*

Reproductive toxicity studies look at the effects of the drug on fertility and on embryonic and post-natal development. In general, reproductive toxicity studies must be completed before a drug can be administered to women of child-bearing age.

- *Carcinogenicity Studies*

Carcinogenicity studies are usually needed only for drugs intended for chronic or recurring conditions. They are time consuming and expensive, and must be planned for early in the preclinical testing process.

- *Toxicokinetic Studies*

These are typically similar in design to PK/ADME studies except that they use much higher dose levels. They examine the effects of toxic doses of the drug and help estimate the clinical margin of safety.

Toxicity testing of new compounds is essential for drug development. The preclinical toxicity testing on various biological systems reveals the species-, organ- and dose- specific toxic effects of an investigational product. Figure 6 presents the critical safety aspect or questions of drug development (based on Pritchard et al., 2003) that need to be addressed during the stages and gates of the innovation model (see Figure 3). Tables 2 and 3 present in vitro and *in-vivo* toxicity studies applicable for establishing safety of new compounds in the different stages of the innovation process. By using this innovation approach, safety questions and toxicity tests are presented in a structured way and the critical question in each gate are highlighted. Hence, this approach could also be of relevance for addressing safety aspect for the development of MNM. These are the first ideas of putting the toxicity tests in an innovation model where further development of safety aspects and toxicity tests in this new

innovation model is warranted. Further development of toxicity testing in a Safe by Design concept will be addressed in deliverable 6.4.

Figure 6. Drug development stages and critical safety questions in the innovation model

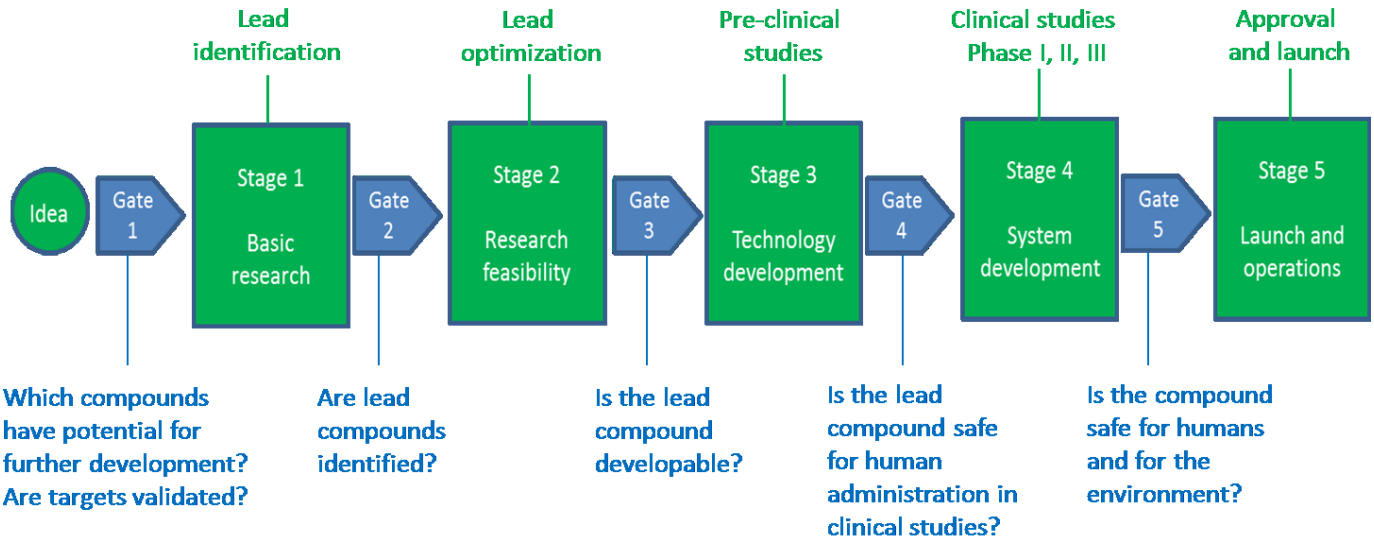


Table 2. Overview of toxicity testing during drug development from stage 1 to gate 3 (Adapted from Pritchard et al., 2003)

Stage 1	Gate 2	Stage 2	Gate 3
Stage activities needed for answering the questions in the next gate	Main questions to be answered before moving to the next stage	Stage activities needed for answering the questions in the next gate	Main questions to be answered before moving to the next stage
Lead identification	**Are lead compounds identified?**	**Lead optimization** (depending on administration route)	**Is the lead compound developable?**
-Pharmaceutical development assessment: solubility, stability. -Bioanalytical assay development. -Cross-validation in rat and dog plasma.	-Can the drug candidate be measured and is it stable in biological matrices?	-*In vitro* metabolism studies: Metabolic stability in hepatocytes, *in vitro* inhibition of CYP450 enzymes across different species, MDCK and/or Caco-2 cell permeability.	-Does the drug candidate have reasonable metabolic stability? -What are the metabolites and are they active, possibly even a better drug candidates? -Are there species differences in metabolism?
-Chemical synthesis process assessment. -Assay development for API purity. -Chemical stability assessment. -Generation of API certificate of analysis.	-Can active pharmaceutical ingredient (API) be synthesized at reasonable costs? -Is the API stable after synthesis?	-*In vivo* intravenous or oral dose pharmacokinetics in rat and dog.	-Does the drug have sufficient oral bioavailability and persistence in the bodies of animal models?
-Pre-formulation development testing. -Assay development for purity and content formulation product.	-Can the drug be formulated for use in animal toxicology studies and early human studies?	-Ames test (bacterial mutation), Mouse lymphoma assay .	-Is the drug mutagenic or cytotoxic *in vitro?*
		-*In vivo* dose finding tests: Single (acute) dosing in mouse and rat, repeated dose range finder and toxicokinetics in rat and dog.	-What is the maximum tolerated dose (MTD) and dose-limiting toxicity? -What is the Multiple intravenous administration tolerated dose (MTMD)?
		-hERG-K+ assay (*in vitro* electrophysiology study using Chinese hamster ovary cells)	-Is the compound likely to induce tachycardia (Torsade de Pointes)?

Table 3. Overview of toxicity testing during drug development from stage 3 to gate 5.

Stage 3	Gate 4	Stage 4	Gate 5
Stage activities needed for answering the questions in the next gate	Main questions to be answered before moving to the next stage	Stage activities needed for answering the questions in the next gate	Main questions to be answered before moving to the next stage
Pre-clinical studies	**Is the lead compound safe for human administration in clinical studies?**	**Clinical studies** **Phase I, II, III**	**Is the compound safe for humans and for the environment?**
-Formulation development. -Define release GMP testing criteria for content and purity. -Define formulation stability. -Establish clinical trial material release specifications.	-Can the drug be reliably prepared and formulated according to Good Manufacturing Practice guidelines that regulate drug material prepared for human use?	-*In vivo* long-term toxicity (6-m to 1-y) in a rodent and a non-rodent species.	-What toxicology arises following long-term administration of the drug?
-Cytochrome protein inhibition kinetic studies. Induction studies with human cultured hepatocytes. -Effects on reporter gene constructs.	-Is there a potential for clinical drug-drug interactions (e.g. enzyme inhibition or induction?)	-Lifetime exposure studies in rat and/or mouse.	-Is the drug carcinogenic?
-*In vitro* studies in plasma or serum of relevant species including human, that has been seeded with radiolabelled drug and free drug separated from bound drug using equilibrium dialysis, ultrafiltration, or chromatography. -*In vitro* studies in blood seeded with radiolabelled drug and erythrocytes separated by centrifugation.	-Does the drug bind to plasma proteins and erythrocytes? If so, how does this affect interpretation of pharmacokinetic data derived from concentration measurements of parent drug in plasma?	-Characterization of complete metabolic profile in humans and animal species used in toxicology testing. Radiolabelled drug often used to trace drug products.	-What is the complete metabolic fate of the drug? -Is there a contribution to efficacy or toxicity by active drug metabolites?

Stage 3	Gate 4	Stage 4	Gate 5
-*In vivo* dose-response studies (14-days to 3-months animal studies with toxicokinetics, maximum no-effect dose), in rats and in dogs or monkeys.	-What is the maximum no-effect dose following repeated dosing? -Is there dose-related exposure ? -What organs are affected by repeated dosing? -What is the safety margin?	-Identification of drug-metabolizing enzymes responsible for new chemical entity metabolism. -*In vitro* drug-drug interaction studies using human enzymes.	-What potential drug-drug interactions can be excluded based on knowledge of the drug's interaction with human drug-metabolizing enzymes or membrane transporters?
-*In vivo* pharmacokinetics: Excretion balance and metabolite identification studies in rat and in dog/monkey including identification of cytochrome P450 enzymes, and quantitative tissue distribution. -*In vitro* drug metabolism studies using human microsomes and/or cytochrome protein expression systems.	-Where does the drug go in the body: how long does it or its metabolites stay there, and by which routes are it and its metabolites excreted? -What enzymes are involved in the drug's metabolism?	-Clinical studies	-Is the drug efficient?
-*In vivo* telemetry studies in animals evaluating alterations in cardiac electrophysiology and cardiovascular vital signs. -Action potential duration using isolated rabbit Purkinje fibres.	-Does the drug produce any cardiovascular effects to cardiac conductance?	**Environmental risk assessment (EMA, 2006):**	
-Irwin behaviour test in rats (*in vivo)*.	-Are there any effects on behaviour?	-Biodegradability test (OECD 301)	-Is the drug biodegradable?
-*In vivo* Rat pulmonary function evaluation.	-Are there any effects on pulmonary function?	-log Kow test -Adsorption-desorption. (OECD 106/121)	-Is the drug likely to bioaccumulate?
-*In vivo* bone marrow micronucleus test in rodents.	-Is the drug genotoxic?	-Aerobic and anaerobic transformation in aquatic sediment systems (OECD 308)	-Is the drug transformed in sediment?
-Reproductive toxicology studies in rats	-Does the drug affect reproductive performance in female rats?	-Algae growth inhibition (OECD 201) -Daphnia reproduction test (OECD 211) -Early life stage toxicity test in fish (OECD 210)	-Are there toxic effects in aquatic organism? -What is the Predicted No Effect Concentration (PNEC)?

Stage 3	Gate 4	Stage 4	Gate 5
		-Activated Sludge respiration inhibition test (OECD 209)	
-Reproductive toxicology studies in rats and rabbits.	-Is there evidence of teratogenicity, mutagenicity or embryo toxicity *in vivo* in rodents?	-Aerobic and anaerobic transformation in soil (OECD 307)	-What is the environmental fate of the drug in terrestrial environment?
-Injection site irritation studies. -Gastrointestinal motility and gastric irritation studies in rats.	-Does the drug irritate the gastrointestinal tract or other sites of administration?	-Soil microorganisms Nitrogen transformation test (OECD 216) -Terrestrial plants growth test (OECD 208) -Earthworm acute toxicity test (OECD 207) *Collembola* reproduction test (ISO 11267)	-Are there toxic effects in terrestrial organisms?

2.3.4 **Regulatory aspects**

The European Medicines Agency is not solely the guidance agency on the drug development process in conjunction with the national notified bodies but also offers scientific advice to support the qualification of innovative development methods for a specific intended use in the context of research and development into pharmaceuticals. Advices are given by the Committee for Medicinal Products for Human Use (CHMP) on the basis of recommendations by the Scientific Advice Working Party (SAWP). This qualification process leads to a CHMP qualification opinion or CHMP qualification advice. EMA is publishing all the committee evaluation and assessment in the format of guidance, reflection papers, and relevant guideline documents

Regarding the regulatory development of drugs with nanotechnology (called nanomedicine), EMA has come forward with several reflection and guidance papers on the matter relevant to the development of medicinal product and medical devices. The development of medicines using newer, innovative nanotechnology techniques may raise new challenges for the Agency in the future. These include discussions on whether the current regulatory framework is appropriate for these medicines and whether existing guidelines and requirements on the way the medicines are assessed and monitored are adequate.

Recommendations from the CHMP (expert group on nanomedicines since 2009) have already led to the approval of a number of medicines based on nanotechnology. These include medicines containing:

- liposomes (microscopic fatty structures containing the active substance), such as Caelyx (doxorubicin), Mepact (mifamurtide) and Myocet (doxorubicin);
- nano-scale particles of the active substance, such as Abraxane (paclitaxel), Emend (aprepitant) and Rapamune (sirolimus).

An overview of the initiatives taken by European Union (EU) regulators in relation to the development and evaluation of nanomedicines and nanosimilars was published, where the regulatory challenges and perspectives in this field are presented (Ehmann et al., 2013).

Regulatory Standards

Preclinical studies are conducted according to good laboratory practice (GLP) guidelines, which regulate how laboratory studies are performed. Clinical trials are conducted according to good clinical practice (GCP) guidelines, which are internationally required quality and safety standards for designing, conducting and reporting clinical trials. GCP-compliant clinical trials are essential to ensure the rights and safety of clinical trial subjects. These standards are subject to inspection by regulatory agencies at any time; regulatory agencies have the right to halt ongoing clinical studies if they have concerns that the studies are not GCP-compliant. Finally drug manufacturing is done according to good manufacturing practice (GMP) guidelines, which dictates the standards for manufacturing and quality control of pharmaceutical products. This is also subject to regulatory inspection. Where applicable, adherence and compliance to International Organization for Standardization (ISO), European Committee for Standardization (CEN) should be followed. Where applicable, specific international or national and directives standards should be also adhered.

Regulatory Submission/Approval

Compliance to regulatory requirements from Food and Drug Administration (FDA) and European Medicine Agencies (EMA) should be taken into account during the development process. Particular attention should be placed into the categorization of the product within each agency as previously stated and as is also available at their respective website (www.fda.gov, www.ema.europa.eu)

Once the phase III studies have completed and delivered a positive outcome, compilation of the data to submit to the regulatory agencies starts. This usually takes several months and can be done by one region at a time, e.g. in the United States, or could be done globally, targeting major regions simultaneously. Classically, the major markets include the United States, the European Union and Japan. However, recently more attention is given to the 'emerging markets' such as Latin America, India and China, amongst others. As for the United States, a routine New Drug Application 'NDA' can take up to 15 months for review. However, in cases of particularly high medical need or in areas lacking treatments (e.g. oncology and human immunodeficiency virus), an expedited review can be granted. If the new drug is a biologic, then a biologic license application 'BLA' rather than a 'NDA', is submitted. In Europe, the sponsor submits a marketing authorization application (MAA), which could be granted either under the centralised procedure (valid for the entire community market) or through the mutual recognition process. During the review by the regulatory agencies, questions are referred back to the sponsor. To facilitate the review process, the

sponsor will typically establish a rapid response team to coordinate the responses to the authority. Drug label negotiations take place during the review process. Regulatory agencies could request post-approval studies from the drug companies to address any safety concerns that the regulatory agencies may have. At the same time, the drug company will have presented its plans to detect, assess and report adverse events. Pharmacovigilance is the term used in Europe describing the ongoing evaluation of the safety of the drug in the post-marketing period; it is a requirement that all pharmaceutical companies with a post-marketed product must comply to. The drug company will also provide periodic safety update reports on the new drug after its approval. Post-marketing or safety surveillance trials are sometimes referred to as phase IV clinical trials. Harmful effects discovered during phase IV trials can lead to the withdrawal of the drug from the market.

2.4 Manufactured nanomaterial development

Nanotechnologies are becoming a substantial part of society and indeed already a multitude of nanotechnology products, or at least products with a nano-based claim, are commercially available (Berube et al., 2005). Nanotechnologies include the development and production of nanosized engineered particles, fibres, coatings, etc., collectively referred to as nanomaterials. Similar to other chemical substances, society, governments and industry alike want to assure that these new products can be used safely. In case risk assessments indicate the unacceptable probability of adverse effects, risk management measures should be taken to protect the environment and human health.

Risks of conventional chemicals are regulated in existing national and international regulatory frameworks. Nanomaterials are often praised for their "new and unique" properties. However, because of these new properties, nanomaterials are also likely to differ from their conventional chemical equivalents with respect to their behaviour in the environment and their kinetic and toxic properties. This raises concerns in connection to their widespread use, as this leads to an increase of exposure to these nanomaterials for humans as well as the environment. As legislation lags behind technological developments (Choi et al., 2009), additional (data) requirements for risk assessment of nanomaterials are yet to be formulated in existing regulatory frameworks. In case regulatory risk assessment procedures are adapted for nanomaterials, it is required that nanomaterials can be clearly and unambiguously identified.

In this chapter, the development of MNMs will be described with a focus on toxicity tests, followed by the requirement for MNM in REACH.

2.4.1 **Manufactured nanomaterial development process**

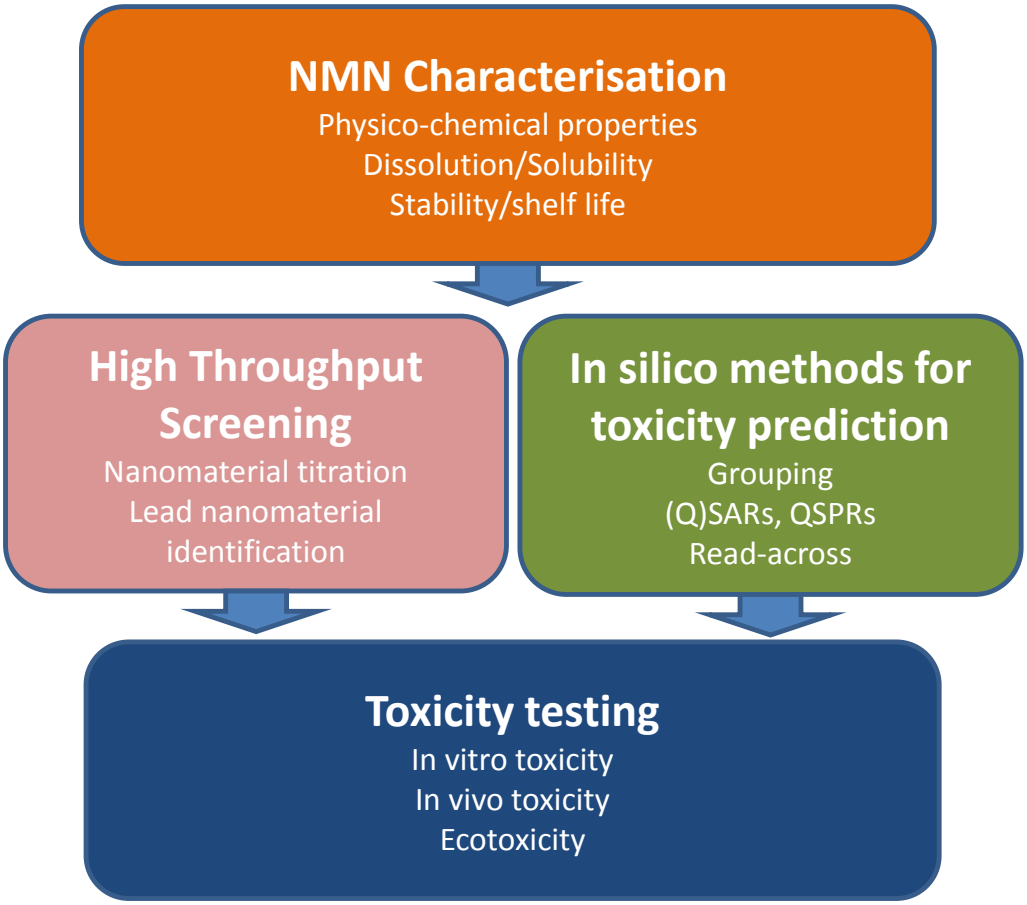
The production of manufactured nanomaterials (MNMs) is a scientific breakthrough in material design and the development of new products. Besides the successful implementation of nanotechnology, it is important to consider the possible environmental health and safety impact as a result of the novel physicochemical properties that could generate hazardous biological outcomes. In order to investigate potential environmental health and safety, information on toxicity of MNMs has to be generated. Here, we describe four key steps during the MNM development that may be of importance to predict and assess toxicity in an efficient way: characterisation of the MNM, high-throughput screening, *in silico* toxicity prediction methods and toxicity testing (see Figure 7).

The developmental process of MNMs starts with the characterisation of the MNM, which includes both the characterisation of physicochemical properties and the solubility of the MNM. Once the MNM has been characterized, both high-throughput screening and *in silico* methods may be used for further development. HTS is used to rapidly and inexpensively predict the effects of MNMs when exposed to suitably chosen cellular models. This can be part of single or multiple cytotoxicity screening with multiple endpoints and timepoints. HTS titration then allow for the lead MNM properties, timepoints or doses to be utilised further with more sensitive assays or *in vivo* as shown in figure 7.

By using *in silico* methods, toxicity of MNMs can be predicted by using grouping methods, quantitative structure-activity relationship (QSAR) methods and/or read-across.

Finally, multiple toxicity assays tests are then performed to investigate cellular interaction (in vitro), organs and tissue toxicity (in vivo) and also environmental ecotoxicity (flora and fauna). Information obtained from the HTS and in silico tests is used for more focused toxicity testing, thereby reducing the number of animals that have to be used.

Figure 7. Schematic approach of MNM development process



Characterisation of MNMs

In order to understand the characteristics of nanomaterials that can contribute to toxicity, they are first assessed in the as-synthesized form, prior to use in in vitro systems and after dispersion in the appropriate media. The physico-chemical properties of the MNM provide qualitative and quantitative information on target effects and mode(s) of action, but also determine its behaviour in the test environment and possible interaction with test constituents, which may alter the test outcomes. Relevant physico-chemical properties include aggregation/agglomeration, water solubility, dispersion stability, dustiness, crystalline phase and crystallite size, particle size distribution, specific surface area and surface chemistry, porosity, zeta potential (surface charge), photocatalytic activity, redox potential and radical formation potential. Properties that may influence exposure and availability (e.g., solubility and dispersability, forming of aggregates or agglomerates) should preferably be monitored during the course of the experiment and in the intended matrix (e.g. dispersed in a liquid, gel or other) as well as in the as-synthesized form. The applicability of standard

methods for measuring physico-chemical properties in nanomaterials is currently under research by the OECD.

Dissolution tests of MNMs

The dissolution test for MNMs is of utmost importance as the dissolution, leading to complete or (most likely) to partial release of the initial forming elements (*e.g.*, metal salts) and/or of secondary unknown substances, strongly may impact on MNM exposure and induced hazard. It may provide information on accumulation/persistency of MNMs along with the identification of other physical forms arising from nanoparticle degradation (such as secondary soluble products, colloidal-solvent complexes, colloidal-protein complexes, etc.) to which humans and environment may be exposed. Not only, it may help interpreting the biological response upon MNM exposure, defining fundamental concepts which are at the basis of the identification of the risk hazard as i) cellular entry modes of MNMs; ii) effective cellular dose, iii) toxicity and associated mechanisms. Definitely, the dissolution test is related to the evaluation of MNMs safety.

Most of the standard dissolution methods and apparatus used for drug development are inappropriate for measuring dissolution of MNMs since they are historically designed for specific dosage forms and they use large volume of media unabling the separation of the solid nanoparticles from the released soluble molecules (especially for smaller sized NPs).

Text box 5. Dissolution testing

According to the IUPAC definition, solubility of a solute is the analytical composition of a saturated solution, expressed in terms of the proportion of the designated solute in a designated solvent. It may be expressed as concentration, molality, mole fraction, mole ratio, etc. (IUPAC, 1987). To perform the solubility test, physiological temperature (37 °C) and pHs in the range of 1 - 7.5 are typically required at saturation conditions. Solubility is a physical parameter fundamental for drug development. Being a thermodynamically driven process, it is sensible to many experimental factors so that its measurement may result particularly challenging at the stage of drug pre-formulation (typically characterized by few amounts of lead compounds). By measuring dissolution of a solute into a solvent at non-equilibrium conditions, the dissolution testing may be therefore a good substitute. Beyond the physical characterization of drugs at the lead developmental research stage, another important utility of the test is providing a drug release profile using *in vivo* like conditions (gastric or intestinal pH and relative molecular compositions) at the stages of drug formulation development and quality control of marketed dosage forms, respectively. Noteworthy, the validated dissolution test represents a good mean to relate *in vitro*/*in vivo* efficacy (or toxicity) (IVIVC) for a given drug/formulation. It is for instance employed by BCS (Biopharmaceutics Classification System) as a predictive tool of the *in vivo* behaviour of active compounds in immediate release solid oral dosage forms (IR). Such approach may also allow to grouping substances with similar solubility/dissolution and intestinal permeability properties by read-across concepts (Biowaiver extension) (Yu et al., 2002).

Regarding the dissolution test and its role on functionality, recently, it has been demonstrated that the dissolution rate of NPs is linked to their functionality in vitro. In particular, for many classes of metal containing nanoparticles, it has been shown a common mechanism of toxicity (LETH mechanism), which is explained by the rapid entrapment of NPs in the lysosomes with a consequent enhanced release of the constituent metal toxic ions (Sabella et al., 2014; Guarnieri et al., 2014). The LETH mechanisms together with others recently explained mechanisms (Wang et al., 2013; Xia et al., 2008) might contribute for the production of safe-by-design NPs along with determining the different decision activities *per* stage-gate. Not only, as the dissolution test may affect the bioavailability of the MNMs, this will also have a tremendous impact on the evaluation of MNM induced hazard. Indeed, the released ions/soluble complexes may be the species producing the major toxicity. Furthermore, as cells rapidly take up colloidal NPs by energy-mediated processes as opposite for ion molecules, this will definitely change the effective dose as well as the cellular entry modes, which will be differently depending on the physical status in which NPs are present in the biological media (colloidal or soluble molecules). All these aspects are also fundamentals for the development of validated test for novel MNMs in a nanotechnology based innovation process.

High-throughput screening for MNMs

High Throughput Screening (HTS) methods and High Content Screening (HCS) methods can be used to test large quantities of compounds for their toxicological potential or possible mechanisms of action, focusing on one single mechanism per test. There are two types of HTS assays: functional assays, which measure the compound's ability to interfere with the function of a target protein, and non-functional assays, which merely measure the binding of a compound to a target protein (Szymanski et al., 2012; Nel, 2013). HTS methods are attractive in that they are fast, cheap, easily reproducible, and that they reduce the number of animals needed for toxicity testing by eliminating animal studies or providing specific targets for further animal or *in vitro* studies. In other words, HTS techniques are very well suitable for a quick hazard screening. Disadvantages of HTS methods include that they have limited predictability for human toxicology: they are not suitable for human dose-response extrapolation and are not predictive for chronic toxicity [ref: ISO]. With the aim of approaching the complex *in vivo* situation more closely, HTS assays have recently been developed using whole animal systems such as yeast, nematodes, and zebra fish (Szymanski et al., 2012).

HTS techniques are especially attractive for the safety evaluation in nanotechnology, because of the enormous variety of nanomaterials that exists (for example, Coccini et al. (2013) reported that for carbon nanotubes only, already 50 000 different variants are being produced). To test all nanomaterials currently on the market by conventional safety evaluation would take between an estimated € 200 million and € 1 billion and 34-53 years (Hartung 2010). Obviously, it is simply impossible to test every manufactured nanomaterial on a case-by-case basis. On the other hand, grouping of nanomaterials based on e.g. structure-activity relationships (SAR) is not as straightforward as for conventional chemicals. Therefore, quick screening of large amounts of nanomaterials by HTS techniques would be of great value.

In silico methods for toxicity prediction

After sufficient information is obtained on the characteristics of the nanomaterial, *in vitro* measurements commence, such as concentration-dependent effects on viability. The results of such preliminary studies can then be examined in animal systems (*in vivo*) for their effects on immune responses or on translocation to other areas after dermal, inhalation, or oral uptake. Once an adequate amount of data are collected, predictive modeling through computer-based approaches can be used to extrapolate the *in vitro* results to *in vivo* situations (Eisenbrand et al., 2002). Toxicokinetic modeling describes the absorption, distribution, metabolism and elimination of xenobiotics within an organism, as a function of dose and time. Toxicokinetic models can be divided into two main categories, namely, data-based compartmental models and physiologically-based compartmental models. Other quantitative structure–activity relationship (QSAR) models have been explored for structurally-related materials. However, many challenges still remain, including predicting the chronic effects that lead to conditions such as cancer, hematotoxicity, hepatotoxicity, lung fibrosis, nephrotoxicity and neurotoxicity, on the basis of in vitro studies. Additionally, most cellular responses are dependent upon dose and exposure time, where a low dose over a long period of time may result in an adaptive or even beneficial/protective effect. After careful consideration, the implications of the research outcome can be used to set safe limits for exposure in the work environment, in consumer products and in environmental waste. How the nanomaterials are distributed, accumulate and persist in the environment, are also matters of great concern.

2.4.2 **Toxicity tests for MNMs**

The specific magnetic, catalytic, optical, electrical or mechanical properties of nanomaterials that exist as a result of the small size and large surface area, may add to potential toxicity. Furthermore, they may influence fate and exposure conditions not only in humans and in the environment, but also in toxicity testing.

Both *in vitro* cell culture and animal studies are being used to evaluate nanomaterials for their toxicity or potential to induce cell death (Barile, 1994). In general, *in vitro* assays consist of subcellular systems (i.e., macromolecules, organelles), cellular systems (i.e., individual cells, coculture, barrier systems) and whole tissues (i.e., organs, slices, explants). The use of relatively simple *in vitro* models with endpoints that reveal a general mechanism of toxicity can be a basis for further assessment of the potential risk of exposure to nanomaterials. The toxicity data obtained from *in vitro* systems has been used to screen, rank and predict the acute hazards and mechanisms of compound interactions with animals or humans. This "basal toxicity" is defined as the ability of a compound to cause cell death as a consequence of damage to basic cellular functions. It can be used to define the concentration ranges of chemicals or nanomaterials which produce a toxic effect. The data obtained from basal toxicity studies have been found to be in good correlation with acute toxicity in animals and humans after studies involving diverse arrays of chemicals and assay systems (Clemedson et al., 2000). However, kinetic factors and target organ specificity were parameters that weakened the correlation. Therefore, *in vitro* studies are conducted as a starting point and are very useful, because of their ability to rapidly and inexpensively produce results, which may uncover the underlying toxic mechanisms of the selected chemicals, without the use of animals. The limitations of *in vitro* methods include: the transformation or immortalization of the cell lines, which may alter the properties and sensitivities of the cells; selective toxicity, in which some cell types are more sensitive than others; the isolation of the cells from their natural environment; and the difficulty encountered in studying integrated groups of cells or organ systems (Schrand et al., 2012). However, new emerging cell culture models have been also developed based on co-culture and 3D models as presented in Movia et al. (ACS NANO 2012 and Biomaterials 2014).

In conclusion, *in vitro* toxicity models offer rapid and effective end points to assess the toxicity of MNMs. *In vitro* toxicity models also offer the following advantages (a) Mechanism-driven evaluations, (b) Dose-response relationships, (c) Suitable for high-throughput screening, (d) System for studying the structural activity relationships, (e) Identify the mechanisms of toxicity in the absence of physiological and compensatory factors that confound the interpretation of whole animal studies, (f) Efficient and cost-effective, (g) Assist

in designing in-*vivo* animal studies (Arora et al., 2012). Hence, it is essential to confirm the in vitro results using appropriate animal models.

Regulatory guidelines for measurement of toxicities of MNMs are still under development. However, agencies like the scientific committee on emerging and newly-identified health risks of the European Commission (SCENHIR) have provided an opinion on measurement methodology for assessing the risks of the MNMs. Risk assessment tools include *in vitro* toxicity studies, standard regulatory *in vivo* toxicology tests, and Quantitative nanostructure-activity relationship (QSAR) models (SCENIHR, 2007). The organisation for economic co-operation and development (OECD) guideline for the testing of chemicals has been implemented for many toxicological endpoints (OECD, 2009). The OECD working group on nanomaterials has recommended a series of endpoints to be addressed in the safety testing of MNMs. An overview of these endpoints and accompanying test methods are presented in Tables 4 and 5. Evaluation of the OECD test methods for their suitability for nanomaterials is currently ongoing. In addition to the OECD report, scientific literature was screened for additional toxicity tests for nanomaterials that have not been covered by OECD standardized methods and these have been included in the overview.

As a detailed discussion on the testing of physico-chemical properties of MNM is outside the scope of this deliverable, tests for physico-chemical properties are not included in the tables. Instead, for the purpose of this overview it is assumed that physico-chemical properties have been addressed before the toxicological tests presented in Tables 4 and 5 are commenced.

Table 4. Mammalian toxicity endpoints to be addressed in the safety testing of MNMs and their utility for MNMs (OECD, 2009; ISO, 2014; Kroll et al., 2009).

Endpoint	Test	OECD standardized methods	Other possible methods	Remarks on utility for nanomaterials
Acute toxicity	Cytotoxicity	-	• MTT test (measures cell viability through mitochondrial activity) • Neutral Red test (measures cell viability through intact lysosomes) • LDH Release test (measures necrosis) • Annexin V / Propidium Iodide test (measures apoptosis / necrosis) • Caspase-3 test (measures apoptosis)	• MTT test is pH-dependent and metal ions (e.g. Zn2+) interfere with reduction reaction. Nanoparticles may interact with the substrate (e.g. SWCNTs were shown to absorb substrate causing false-negative results) • Neutral Red test is pH-dependent. Nanomaterials may adsorb the neutral red, leading to false-negative results (underestimation of cell viability) • LDH Release test is pH-dependent and metal ions (e.g. Cu) have been shown to inhibit LDH release) • Nanoparticles may cause false-negative apoptosis results and false-positive necrosis results in Annexin V/ Propidium Iodide test (e.g. gold NPs have been shown to bind to the propidium iodide and then "transport it" into intact cells) • Trace metal ions (e.g. Zn2+) inhibit the activity of Caspase-3, leading to false-negative results.
	Stress response (*in vitro)*	-	• ROS determination test using H2DCF-DA • ROS determination test using GSH (intracellular ROS generation)	• H2DCF-DA test is pH-dependent. The deacetylated H2DCF may accumulate in extracellular space and react with catalytically active substances outside the cells >>> thus it could directly react with nanoparticles. Also, nanoparticles may interfere with the

Endpoint	Test	OECD standardized methods	Other possible methods	Remarks on utility for nanomaterials
				fluorescence measurement by absorbing light, leading to false-negative results.
	Inflammatory response (*in vitro)*	-	• Detection of cytokine release by ELISA (inflammatory response)	• Metal oxides and carbon nanoparticles may absorb cytokines. Also, nanoparticles may deplete nutrients or other growth factors in the cell culture media (because of their high adhesive surface area) >>> false-negative results. Furthermore, nanoparticles may be contaminated with e.g. endotoxin >>> false positive results.
	In Vivo acute toxicity testing (LD50, pathology)	• 420: Acute Oral toxicity – Fixed Dose • 423: Acute Oral toxicity – Acute Toxic Class Method • 425: Acute Oral toxicity: Up-and-Down Procedure • 402: Acute Dermal Toxicity • 403: Acute Inhalation Toxicity		• Aggregation or agglomeration may increase nanoparticle uptake by macrophages
	Skin corrosion and skin irritation	• 430: *In vitro* Skin Corrosion: Transcutaneous Electrical Resistance Test • 431: *In vitro* Skin Corrosion: Human Skin Model Test • 435*: In vitro* Membrane Barrier Test Method for Skin Corrosion • 404: Acute Dermal Irritation / Corrosion	• 428: Human skin models • 439: *In vitro* skin irritation: Reconstructed Human *Epidermis* Test Method	

Endpoint	Test	OECD standardized methods	Other possible methods	Remarks on utility for nanomaterials
	Skin sensitisation	• 406: Skin Sensitisation (guinea pig) • 429: Skin Sensitisation: Local Lymph Node Assay (murine)		• Skin penetration by insoluble materials is problematic and could lead to false negative results
	Acute eye irritation **	• 405: Acute Eye Irritation / Corrosion (*in vivo*)		
	Photo toxicity	• 432: *In Vitro* 3T3 NRU Phototoxicity test (using cultured murine cells)		
Repeated dose toxicity	Repeated dose toxicity with oral administration	• 407: Repeated Dose 28-Day Oral Toxicity Study in Rodents • 409: Repeated Dose 90-Day Oral Toxicity Study in Non-Rodents		
	Repeated dose toxicity with dermal administration	• 410: Repeated Dose Dermal Toxicity: 90-Day (rodents and non-rodents)		
	Repeated dose toxicity with inhalation exposure	• 411: Subchronic Inhalation Toxicity: 90-Day • 412: Repeated Dose Inhalation Toxicity: 28/14-Day • 413: Subchronic Inhalation Toxicity: 90-Day		
Chronic toxicity / carcinogenicity	Chronic toxicity and carcinogenicity	• 451: Carcinogenicity Studies (in rodents, oral dosing) • 452: Chronic Toxicity Studies (in rodents) • 422: Combined Repeated Dose Study with the Reproduction / Developmental Toxicity Screening Test • 453: Combined Chronic Toxicity / Carcinogenicity Studies (in rodents)		
Reproductive	One-generation	• 415: One-Generation Reproductive		

Endpoint	Test	OECD standardized methods	Other possible methods	Remarks on utility for nanomaterials
toxicity	reproductive study	Toxicity		
	Modified one-generation reproductive study	• 414: Prenatal Developmental Toxicity Study		
	2-year reproductive toxicity study (additional generation)	• 416: Two-generation Reproduction Toxicity Study		
Developmental toxicity	Developmental toxicity	• 414: Prenatal Developmental Toxicity Study • 421: Reproduction / Developmental Toxicity Screening Test		
Genetic toxicity	Genetic toxicity *in vitro*	• 471: Bacterial Reverse Mutation Test (AMES test) • 473: *In vitro* Mammalian Chromosomal Aberration Test • 476: *In vitro* Mammalian Cell Gene Mutation Test (mouse lymphoma cells)	• 489: *In vivo* Alkaline Mammalian Comet Assay • 482: Unscheduled DNA Synthesis Test with Mammalian Cells *In vitro* • 487: *In vitro* micronucleus assay (MNvit)	• Nanoparticles may not be able to penetrate the bacterial cell wall, leading to false-negative results in the AMES test. • Nanoparticles may interact with the DNA-repair enzyme FPG used in the Comet Assay, leading to false-negative results
	Somatic cell genotoxicity *in vivo* ***	• 475: Mammalian Bone Marrow Chromosomal Aberration Test • 474: Mammalian Erythrocyte Micronucleus Test • 486: Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *in vivo*		
	Germ cell mutagenicity *in vivo* ***	-		

Endpoint	Test	OECD standardized methods	Other possible methods	Remarks on utility for nanomaterials
Pharmacokinetics Toxicokinetics	Radiolabelling (*in vivo*) -			
Other relevant test data (including endpoints not mentioned in OECD report)	Whole Animal Screening Model		• Embryonic Zebrafish Model	• Cost- and time-efficient method with the advantages of whole-animal screening. Drawback: nanoparticles may clog the gills of the zebrafish, thus observed effects may originate from a physical cause rather than a toxicological cause.
	Inflammatory response / Immunotoxicity		• Immunophenotyping (using flow cytometry or immunohistochemistry) • Modular immune *in vitro* construct system (Test series comprising Peripheral Tissue Equivalent and Lymphoid Tissue Equivalent modules) • T-cell dependent antibody response • Natural Killer (NK) cell activity assay • Host resistance study (*in vivo* challenging of rats or mice) • Macrophage and neutrophil function assays (*in vitro, in vivo* or *ex vivo)*	• Could be used as a standard toxicity test
	Barrier perturbation: skin barrier		• 428: Skin Absorption: *In vitro* Method • Franz-type diffusion cells • Saarbrucken penetration model • Animal models (*in vivo* or *in vitro)*	• Dermal uptake is influenced by dispersant • Animal models are less useful, due to differences in permeability and hair follicle density of animal vs human skin

Endpoint	Test	OECD standardized methods	Other possible methods	Remarks on utility for nanomaterials
	Barrier perturbation: pulmonary barrier		• *In vitro* human airway tissue model • 3D-models • Air-liquid interface models	• The *in vitro* human airway tissue model is a commercially available method, that has been tested by multiple laboratories
	Barrier perturbation: blood-brain barrier		• Cell co-cultures	
	Barrier perturbation: placental barrier		• *Ex vivo* human placental perfusion model	
	Neurotoxicity	-	-	

* Numbers refer to OECD Test Guidance documents, unless stated otherwise

** To be performed only in case of absence of skin corrosion

*** To be performed only in case of positive *in vitro* genotoxicity outcome. Preferably to be measured during repeated dose toxicity. Either one of somatic cell genotoxicity or germ cell mutagenicity should be measured, not both.

Table 5. Endpoints to be addressed in the Environmental safety testing of MNMs (OECD, 2009; ISO, 2014)

Endpoint	Test	OECD standardized methods	Other possible methods
Environmental Fate	Dispersion stability in water	-	
	Biotic degradability in different compartments	301: Ready biodegradability 310: Ready biodegradability – CO2 in sealed vessels (Headspace test) 302: Inherent biodegradability 306: Biodegradability in Seawater 309: Aerobic Mineralisation in Surface Water – Simulation Biodegradation Test 304A: Inherent Biodegradability in Soil 307: Aerobic and Anaerobic Transformation in Soil 308: Aerobic and Anaerobic Transformation in Aquatic Sediment Systems 303A: Simulation Test – Aerobic Sewage Treatment: Activated Sludge Units 303B: Simulation Test – Aerobic Sewage Treatment: Biofilms 311: Anaerobic Biodegradability of Organic Compounds in Digested Sludge	
	Identification of degradation products	-	
	Further testing of degradation products (as required)	-	
	Abiotic degradation and fate	111: Hydrolysis as a function of pH 316: Phototransformation of Chemicals in Water – Direct	

		Photolysis
	Adsorption – Desorption	106: Adsorption / Desorption Using a Batch Equilibrium Method
	Adsorption to soil or sediment	-
	Bioaccumulation potential	305: Bioconcentration: Flow-through Fish Test 315: Bioaccumulation in Sediment-dwelling Benthic Oligochaetes
Ecotoxicity / Environmental toxicity. **Relevant tests should be selected according to information on physico-chemical and environmental fate properties.**	Effects on aquatic species	203: Fish, Acute Toxicity Test 204: Fish, Prolonged Toxicity Test: 14-Day study 210: Fish, Early-Life Stage Toxicity Test 212: Fish, Short-term Toxicity Test on Embryo and Sac-fry Stages 215: Fish, Juvenile Growth Test 202: Daphnia sp. Acute Immobilisation Test 211: Daphnia Magna Reproduction Test 201: Freshwater alga and cyanobacteria growth inhibition test
	Effects on sediment species	218: Sediment-Water Chironomid Tox Using Spiked Sediment 219: Sediment-Water Choronimid Tox Using Spiked Water 225: Sediment-Water Lumbriculus Toxicity Test Using Spiked Sediment ISO 10872: Water quality — Determination of the toxic effect of sediment and soil samples on growth, fertility and reproduction of *Caenorhabditis elegans (Nematoda)*

	Effects on soil / terrestrial species	207: Earthworm, Acute Toxicity Test 222: Earthworm Reproduction Test (Eisenia fetida / Eisenia andrei) 220: Enchytraed reproduction test 226: Predatory Mite reproduction test in soil ISO 11267: Springtail reproduction test with *Folsomia candida*	ASTM E2172-01: Standard Guide for Conducting Laboratory Soil Toxicity Tests with the Nematode *Caenorhabditis elegans* ISO 10872: Water quality — Determination of the toxic effect of sediment and soil samples on growth, fertility and reproduction of *Caenorhabditis elegans (Nematoda)*
	Effects on micro-organisms	216: Soil Microorganisms: Nitrogen Transformation Test 217: Soil Microorganisms: Carbon Transformation Test	
	Effects on activated sludge at wastewater treatment plants	209: Activated sludge respiration inhibition test	

Toxicity studies are essential for MNM development. Tables 6 and 7 present *in vitro* and *in vivo* toxicity studies applicable for establishing safety of MNMs (based on ISO, 2014, OECD, 2009 and REACH) at different stages of the innovation process. As mentioned in the drug development chapter, by using this innovation approach, safety questions and toxicity tests are presented in a structured way and the critical question in each gate are highlighted. These are the first ideas of putting the toxicity tests in an innovation model, where further development of safety aspects and toxicity tests in this new innovation model is warranted. Further development of toxicity testing in a Safe by Design concept will be addressed in deliverable 6.4, expected to be finished mid 2015.

Table 6. Overview of toxicity testing during MNM development from stage 1 to gate 3.

Stage 1	Gate 2	Stage 2	Gate 3
Stage activities needed for answering the questions in the next gate	Main questions to be answered before moving to the next stage	Stage activities needed for answering the questions in the next gate	Main questions to be answered before moving to the next stage
-Computational modelling (e.g. QSARs, Read-across)	-Identify initial adverse effects/molecular initiating events -Identify possible "toxicity pathways" considering the exposure routes of concern	-*In vitro* Cytotoxicity -*In vitro* Stress response -*In vitro* Inflammatory response -*In vivo* acute toxicity testing (LD50, pathology) -*In vitro* and *in vivo* Skin corrosion and skin irritation -Skin sensitisation -Acute eye irritation (*in vitro* and *in vivo*) -Phototoxicity	-Assess acute toxicity of the pristine MNM
-Basic Physico-chemical characterization	-Characterization and testing basic physico-chemical properties i.e. dustiness, solubility, etc. -Identify similar physicochemical characteristics with impact on (eco)toxicity	-Toxicokinetics: *in vitro* metabolism studies	-ADME
		-*In vitro* genetic toxicology / mutagenicity test (e.g., Ames test)	-Indication of genetic toxicology
		-Embryonic Zebra fish model (whole animal screening model)	-Identification of target organs / effects
		Environmental fate tests: -Dispersability -Biodegradation, ready biodegradability -Abiotic degradation - Identification of degradation products -Adsorption/Desorption, adsorption to soil or sediment - Simulation testing on degradation in surface water - Simulation testing in soil and sediment	-Environmental fate: Identification of relevant compartments for ecotoxicology

Table 7. Overview of toxicity testing during MNM development from stage 3 to gate 5.

Stage 3	Gate 4	Stage 4	Gate 5
Stage activities needed for answering the questions in the next gate	Main questions to be answered before moving to the next stage	Stage activities needed for answering the questions in the next gate	Main questions to be answered before moving to the next stage
-*In vivo* dose finding and acute toxicity (oral, dermal, inhalation)	-Assess acute toxicity of the MNM in the product	-*In vivo (sub)chronic toxicity* test	-(Sub)chronic toxicity
-*In vivo* repeated dose toxicity (oral, dermal, inhalation)	-Repeated dose toxicity, dose-response	- *In vivo* carcinogenicity test	- Carcinogenicity
		-Two-generation reproductive toxicity study -Somatic cell genotoxicity *(in vivo*) -Germ cell mutagenicity *(in vivo*) -Developmental toxicity tests	-Reproductive, Developmental and Genotoxic effects
		-Barrier perturbation test (skin barrier, pulmonary barrier, blood-brain barrier or placental barrier) - Immunotoxicity - Specific organ toxicity tests (e.g., enzyme inhibition) - Neurotoxicity	- Specific effects, depending on mode of action
- Short-term toxicity to invertebrates (e.g., Daphnia) - Short-term toxicity to fish - Short-term toxicity to plants - Algae growth inhibition test - Short-term toxicity to sediment / benthic organisms - Short-term toxicity to soil / terrestrial organisms - Effects on micro-organisms - Activated sludge (STP) respiration inhibition test	-Short-term environmental toxicity	- Further information on environmental fate, behaviour and degradation products - Bioaccumulation potential (preferably in fish) - Long-term toxicity to invertebrates - Long-term toxicity to fish - Long-term toxicity to (aquatic) plants - Long-term toxicity to sediment organisms - Long-term toxicity to soil / terrestrial organisms -Long-term or reproductive toxicity to birds	-Long-term environmental fate and toxicity

2.4.3 Regulatory aspects for manufactured nanomaterials in REACH

The REACH regulation (EC no 1907/2006), which is short for the Registration, Evaluation and Authorization and Restriction of Chemicals, has entered into force on 1st June 2007 and is dedicated to streamline the former regulatory framework of chemicals in the EU (EU, 2006). Under REACH, manufacturers, importers and downstream users should guarantee that the substances they manufacture, place on the market or use are safe for workers, consumers and the environment. Registrants are obliged to collect all available relevant information on the intrinsic properties of a substance when manufactured or imported at a quantity of 1 tonne per year (t/yr) or more. However, the type and quantity of information that is required as a minimum to meet the obligations of the REACH regulation depends on the tonnage level (≥ 1 t/yr, ≥10 t/yr, ≥ 100 t/yr, ≥ 1000 t/yr or more).

REACH and nanomaterials: a matter of definition

The incremental development of new nanomaterials and their increased used in all sorts of industrial applications and consumer products, in combination with their specific characteristics, have accelerated the need to address the potential risks and hazards of nanomaterials and to develop regulatory frameworks for adequate risk assessment and risk management. The European Commission is convinced that REACH offers the best framework for the (regulatory) risk assessment and safe handling of nanomaterials (EC, 2012). Under REACH a substance is defined as:

'a chemical element and its compounds in the natural state or obtained by any manufacturing process, including any additive necessary to preserve its stability and any impurity deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition' (EC, 2006).

Although not specifically addressed, the safety of nanomaterials should be covered by this definition (Pronk et al., 2009). The general obligations in REACH, such as registration of substances manufactured at 1 tonne or more per year and providing information in the supply chain also apply for nanomaterials as for any other substance. Within REACH there are no provisions yet that specifically deal with the risk management of nanomaterials. Other legislation (e.g. cosmetics, biocides and food-related legislation) make explicit reference to nanomaterials in some parts of the legislative text.

To make the inclusion of nanomaterials within REACH more specific and to leave the option open for a request for additional data requirements for nanomaterials, a harmonized well-accepted definition for nanomaterials is essential (Pronk et al., 2009; Bleeker et al., 2013). To ensure a uniform implementation in different regulatory frameworks one single legally binding definition (i.e. a horizontal definition) is preferred. The primary focus of such a definition should be the identification of a substance as a nanomaterial. In the 13th meeting of the REACH Competent Authorities Sub-Group on Nanomaterials (CASG Nano) in May 2014[1] the EC proposed to include the following definition for nanomaterials in Annex VI of the REACH regulation:

'*a nanomaterial is a natural 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 nm-100 nm'.*

This definition is the same as the one used in the Biocides Regulation (EC/528/2012) and in accordance with the European Commission Recommendation of 18 October 2011 on the definition of nanomaterials (EU, 2011). The definition in the recommendation will be reviewed by December 2014, which may influence the definition to be used in the REACH regulation.

Data requirements for nanomaterials

Nanomaterials have specific characteristics that distinguish them from non-nanomaterials. Some properties of nanomaterials are specifically associated with hazards for human and environmental health. To identify and characterize nanomaterials in several critical life cycle stages additional information on various properties is needed. Also for risk assessment purposes, additional information on human and environmental toxicity and exposure is essential. This recognition initiated three REACH Implementation Projects on Nanomaterials (RIPoNs)2, leading to appendices to the REACH Guidance. Nevertheless, it was recognised that further requirements for nanomaterials should be included in the legal text as well (i.e. in the REACH Annexes). In 2013, the Danish Environmental Protection Agency (Danish EPA) has

[1] In this meeting the EC presented a proposal for amendment of the Annexes to REACH for nanomaterials (http://*chemicalwatch.com/downloads/13thCASGagenda.pdf).*

[2] More information on the RIP-oNs can be found at the website of the European Commission: http://ec.europa.eu/environment/chemicals/nanotech/reach-clp/ripon_en.htm

published a proposal for the regulatory information requirements for nanomaterials, based on scientific motivation for the request for additional data, availability of test methods and consensus among experts (Christensen and Larsen, 2013). This proposal was used in a report by RIVM (Bleeker et al., 2013) describing an exploration of options to amend existing EU regulations in order to regulate nanomaterials. Based on both documents, data requirements for the identification, characterization and toxicological risk assessment of nanomaterials are identified, as further described in section A and B.

The default in REACH is to register nanomaterials as a specific form of a bulk substance ('one substance - one registration principle'). Only in specific cases nanomaterials may be registered as a distinct substance. Nevertheless, also when different forms of a substance are registered in one dossier, for each specific form the safe use should be guaranteed, if necessary supported by additional data. It is evident that for some nanomaterials specific testing may be necessary to ensure their safety.

A. Identification and characterization of nanomaterials

For identification and characterization purposes information on several morphological and physicochemical properties is required in REACH (see table 8).

In table 8 the substance identification and characterization parameters required in REACH are assessed for their applicability for nanomaterials. For nanomaterials, many of the described parameters read-across of the data obtained for the bulk version of the substance could be sufficient. Several parameters, however, need adaptation and some are not explicitly mentioned in REACH and need to be added (highlighted with asterisks).

Due to significant knowledge gaps associated with the identification of nanomaterial properties, it remains subject of debate whether existing test guidelines are suitable for nanomaterials. According to the OECD 2009 review (OECD, 2009), the majority of the endpoints and the test guidelines are generally considered acceptable to address nanomaterials. It is recognized that some methodological challenges still need to be clarified, specifically sample preparation and dosimetry issues (see also OECD 2012). Table 8 includes remarks on the applicability of existing test methodologies and guidance documents for nanomaterials (based on OECD, 2009; Christensen and Larsen, 2013 and Bleeker, 2013).

B. Data requirements for the toxicological risk assessment

For toxicological risk assessment purposes further information on nanomaterials is necessary, including:

- Toxicological information, including extra genotoxicity tests, a focus on the inhalation route, and adaptation of repeated dose testing regulations
- Ecotoxicological information, including sediment and terrestrial toxicity testing, as well as acute and particularly chronic testing
- Information on exposure, risk characterisation and risk management, including exposure and release information, identification and characterisation of nanomaterials in various life cycle stages, and nanospecific risk management measures.
- A list of required toxicological information is shown in Tables 9 and 10, including a short assessment of the existing test methodologies and guidance documents (OECD, 2009, Christensen and Larsen, 2013 and Bleeker, 2013). Especially for inhalational testing, environmental fate assessment and ecotoxicity further method development and inclusion of additional examinations should be considered (Christensen and Larsen, 2013, Bleeker et al., 2013).

REACH and nanomaterials: tonnage levels

As illustrated in the previous section, REACH could generally be used to obtain the required nanomaterial-specific safety information. As shown in Tables 8 and 9, for some parameters adaptations are needed and existing test methodologies and guidance documents require further development.

In general, the current tonnage levels within REACH are considered too high for nanomaterials to fill the data gaps in relation to the risk assessment and the risk management of nanomaterials, especially for the ecotoxicity parameters, as shown in Table 10 (Bleeker et al., 2013). Moreover, many of the known nanomaterials are produced below the lowest REACH cut-off point of 1 t/yr. As a result, for these nanomaterials there is as yet no registration obligation under REACH, unless they are included under the registration of a non-nanomaterial. Lowering the established tonnage levels for nanomaterials requires further adaptation of the main text of REACH, which is a policy issue and beyond the scope of this document.

Table 8. Substance identification and characterization parameters required in REACH

2	IDENTIFICATION OF THE SUBSTANCE	Remarks
2.1	**Name or other identifier of each substance**	Discussion whether each nanomaterial is considered a substance on its own or is a specific form of one substance has impact on these parameters.
2.1.1	Name(s) in the IUPAC nomenclature or other international chemical name(s)*	
2.1.2	Other names (usual name, trade name, abbreviation)*	
2.1.3	EINECS or ELINCS number (if available and appropriate)	
2.1.4	CAS name and CAS number (if available)	
2.1.5	Other identity code (if available)*	
2.2	**INFORMATION RELATED TO MOLECULAR AND STRUCTURAL FORMULA OF EACH SUBSTANCE**	
2.2.1	Molecular and structural formula (including SMILES notation, if available)	Discussion whether each nanomaterial is considered a substance on its own or is a specific form of one substance has impact on these parameters.
2.2.2	Information on optical activity and typical ratio of (stereo) isomer (if applicable and appropriate)	
2.2.3	Molecular weight or molecular weight range	
2.3	**COMPOSITION OF EACH SUBSTANCE**	
2.3.1	Degree of purity (%)	These parameters may specifically affect properties Spectral data (2.3.5) are particularly of interest for nanomaterials with surface modifications. Existing test methodologies and guidance are considered relevant for nanomaterials.
2.3.2	Nature of impurities, including isomers and by-products	
2.3.3	Percentage of (significant) main impurities	
2.3.4	Nature and order of magnitude (... ppm, ... %) of any additives (e.g. stabilizing agents or inhibitors)*	
2.3.5	Spectral data (ultra-violet, infra-red, nuclear magnetic resonance or mass spectrum)	
2.3.6	High-performance liquid chromatogram, gas chromatogram	

7	PHYSICOCHEMICAL PROPERTIES	Tonnage level	Remarks
7.1	State of substance at 20 °C and 101.3 kPa	≥ 1 tonne/year	IUCLID 5.2 offers an option to indicate that substance is registered as a 'nanomaterial', but this option is rarely used. Existing test methodologies and guidance are considered relevant for nanomaterials.
7.2	Melting / freezing point	≥ 1 tonne/year	Used for waiving if melting point > 300 °C, which is the case for many nanomaterials. Existing test methodologies and guidance are considered relevant for nanomaterials.
7.3	Boiling point	≥ 1 tonne/year	Under REACH this parameter can be waived if melting point is >300 °C and would therefore not be needed for the current generation of nanomaterials. (Christensen and Larsen, 2013)
7.4	Relative density/particle concentration	≥ 1 tonne/year	Existing test methodologies and guidance are considered relevant for nanomaterials.

7.5	Vapour pressure	≥ 1 tonne/year	Guidance needed to specify which surface modified nanomaterial should be addressed. Existing test methodologies and guidance are considered relevant for nanomaterials.
7.6	Surface tension* Specific surface area by volume* Surface charge / zeta potential / isoelectric point* Other surface properties (surface structure, surface acidity, surface energy, surface reactivity – incl. surface chemistry)*	≥ 1 tonne/year	May specifically affect properties. Further study required to determine the need to include surface tension in basic list of requirements. Crystal structure is relevant for metal-based nanomaterials. Existing test methodologies and guidance are generally considered relevant for nanomaterials, but for some parameters further method development is still required. For surface tension, OECD noted that current OECD guidelines are applicable for some nanomaterials under specific circumstances (OECD, 2009)
7.7	Water solubility* Dissolution kinetics* Dispersibility / dispersion stability*	≥ 1 tonne/year	Terminology solubility/dispersibility/dissolution requires further clarification. Relevance of existing test methodologies and guidance should be re-assessed based on consensus on terminology. Currently OECD is updating and developing guidelines and guidance documents for these parameters
7.8	Partition coefficient n-octanol / water* Fat solubility / oleophilicity*	≥ 1 tonne/year	Potential waiving for nanomaterials surface modified with inorganic groups should be addressed (Christensen and Larsen, 2013). A general method to address hydrophilicity/hydrophobicity of nanomaterials is preferred (Christensen and Larsen, 2013).
7.9	Flash-point	≥ 1 tonne/year	Only relevant for liquids
7.10	Flammability	≥ 1 tonne/year	Existing test methodologies and guidance are considered relevant for nanomaterials.
7.11	Explosive properties	≥ 1 tonne/year	
7.12	Self-ignition temperature	≥ 1 tonne/year	
7.13	Oxidising properties Catalytic properties / photocatalytic properties / radical formation potential*	≥ 1 tonne/year	Need for method development.
7.14	Granulometry, specific: particle size distribution * Aggregation and agglomeration behaviour* Appearance / morphology (shape, aspect ratio)* Dustiness*	≥ 1 tonne/year	May specifically affect properties. Proposed test methodologies are still subject to debate, and no single method is applicable for all nanomaterials. Dustiness is relevant for all powder-based nanomaterials, but REACH guidance for measuring dustiness requires updating.
7.15	Stability in organic solvents and identity of relevant degradation products	≥ 100 tonnes/year	Existing test methodologies and guidance are considered relevant for nanomaterials.
7.16	Dissociation constant	≥ 100 tonnes/year	May be relevant for some nanomaterials. Current OECD test guidelines may be applicable to some nanomaterials under some circumstances.
7.17	Viscosity	≥ 100 tonnes/year	Not relevant for nanomaterials

* These parameters need adapted in the current REACH requirements. The parameters without a number are currently not (explicitly) mentioned in the REACH requirements.

Table 9. Toxicological information requirements in REACH

8	TOXICOLOGICAL INFORMATION*	Tonnage level	Remarks
8.1	Skin irritation / corrosion – *in vitro*	≥ 1 tonne/year	Existing *in vitro* and *in vivo* test guidelines are considered applicable for nanomaterials (OECD, 2009).
8.1.1	Skin irritation – *in vivo*	≥ 10 tonnes/year	
8.2	Eye irritation – *in vitro*	≥ 1 tonne/year	
8.2.1	Eye irritation – *in vivo*	≥ 10 tonnes/year	
8.3	Skin sensitisation	≥ 1 tonne/year	Existing test guidelines are considered applicable for nanomaterials (OECD, 2009).
8.4.1	*In vitro* gene mutation study in bacteria**	≥ 1 tonne/year	Focus should be on mammalian (non-bacterial) assays. *In vivo* mutagenicity test guidelines are considered applicable for nanomaterials (OECD, 2009). Further development/validation of assays to test a broad spectrum of mode of actions is required.
8.4.2	*In vitro* cytogenicity study in mammalian cells or *in vitro* micronucleus study**	≥ 10 tonnes/year	
8.4.3	*In vitro* gene mutation study in mammalian cells**	≥ 10 tonnes/year	
8.4	*In vivo* mutagenicity studies**	≥ 100 tonnes/year	
8.5.1	Acute oral toxicity**	≥ 1 tonne/year	For acute toxicity extended examinations (pathology/histology) are preferred. Inhalation route should be the first route of choice, for inhalation extended examinations (inclusion of bronchoalveolar lavage (BAL) and pulmonary cell proliferation) should be considered.
8.5.2	Acute inhalation toxicity**	≥ 10 tonnes/year	
8.5.3	Acute dermal toxicity	≥ 10 tonnes/year	
8.6.1	Short-term repeated dose toxicity study (28 days)**	≥ 10 tonnes/year	Inhalation route should be the first route of choice. Extended examinations are preferred (inflammatory, cardiovascular, neurotoxic and immunotoxic (as summarized in Christensen and Larsen, 2013). Specific caution for poorly soluble particles (lung- overload phenomenon).
8.6.2	Sub-chronic toxicity study (90 days)**	≥ 100 tonnes/year	
8.6.3	Long term toxicity study (≥ 12 months)**	≥ 1000 tonnes/year	
8.6.4	Further studies**	≥ 1000 tonnes/year	
8.7.1	Screening for reproductive / developmental toxicity (OECD 421 or 422)**	≥ 10 tonnes/year	Existing guidelines require modification to inhalational exposure.
8.7.2	Pre-natal developmental toxicity study**	≥ 100 tonnes/year	
8.7.3	Two-generation reproductive toxicity study**	≥ 100 tonnes/year	
8.8.1	Assessment of the toxicokinetic behaviour (ADME) of the substance to the extent that can be derived from the relevant available information**	≥ 10 tonnes/year	Particular concern for distribution into brain and through the placenta. More guidance is needed on method design, potentially on case-by-case basis. Focus on dermal absorption (e.g. cosmetics).
8.9.1	Carcinogenicity**	≥ 1000 tonnes/year	Existing test guidelines are less detailed with regard to investigating neurotoxicity effects than 90 days study (OECD, 2009).
	* In general, monitoring of changes in the physical form and characteristics of nanomaterials during toxicological testing is recommended, as this is instrumental for read-across approaches in the future. ** These parameters (may) need adaptation for nanomaterials		

Table 10. Ecotoxicological information requirements in REACH

9	ECOTOXICOLOGICAL INFORMATION*	Tonnage level	Remarks
9.1.1	Short-term toxicity testing on invertebrates (preferred species Daphnia)**	≥ 1 tonne/year	End-points measured in these tests are relevant and applicable for nanomaterials but development of specific test systems/guidelines, with respect to further markers/parameters for toxicity is required (OECD, 2009). Proposed to include short-term ecotoxicity testing at the lowest tonnage level.
9.1.2	Growth inhibition study aquatic plants (algae preferred)**	≥ 1 tonne/year	
9.1.3	Short-term toxicity testing on fish**	≥ 10 tonnes/year	
9.1.4	Activated sludge respiration inhibition testing	≥ 10 tonnes/year	
9.1.5	Long-term toxicity testing on invertebrates (preferred species Daphnia)**	≥ 100 tonnes/year	End-points measured in these tests are relevant and applicable for nanomaterials but development of specific test systems/guidelines, with respect to further markers/parameters for toxicity is required (OECD, 2009). Proposed to include long-term ecotoxicity testing at a lower tonnage level.
9.1.6	Long-term toxicity testing on fish**	≥ 100 tonnes/year	
9.2.1.1	Biotic degradation – ready biodegradability	≥ 1 tonne/year	OECD concluded that only some of the test guidelines on biotic degradation are applicable to nanomaterials, and only for those of the nanomaterials that contain carbon that can be utilized for microbial growth. Inorganic nanomaterials should not be tested in any of the biotic degradation tests (as summarized in Christensen and Larsen, 2013).
9.2.1.2	Simulation testing on ultimate degradation in surface water	≥ 100 tonnes/year	
9.2.1.3	Soil simulation testing	≥ 100 tonnes/year	
9.2.1.4	Sediment simulation testing	≥ 100 tonnes/year	
9.2.2.1	Abiotic degradation – hydrolysis as function of pH	≥ 10 tonnes/year	Prioritization for nanomaterials that may be subject to hydrolysis (e.g. esters, amide groups etc). OECD test guidelines only relevant for nanomaterials with groups that could be subject to hydrolysis (OECD, 2009). May include testing of photodegradation. For this test, OECD test guidelines are considered applicable (OECD, 2010).

9.2.3	Identification of degradation products	≥ 100 tonnes/year	
9.2	Further biotic degradation	≥ 1000 tonnes/year	
9.3.1	Adsorption / desorption screening study	≥ 10 tonnes/year	Octanol-water partition coefficient (K_{ow}) is not suitable. Distribution coefficient (K_d) should be determined. OECD test guidelines are generally considered applicable, caution for colloid suspensions (OECD, 2009).
9.3.2	Bioaccumulation in aquatic species, preferably fish**	≥ 100 tonnes/year	Log K_{ow} cannot be used for predicting bioaccumulation for insoluble nanomaterials. The OECD test guideline with fish and the OECD test guideline with sediment worms are considered appropriate to generate bioaccumulation data for nanomaterials (OECD, 2009). Preference for lowering the REACH tonnage level for this parameter.
9.3.3	Further information on absorption / desorption**	≥ 100 tonnes/year	
9.3.4	Further information on the environmental fate and behaviour and/or degradation products**	≥ 1000 tonnes/year	
9.4.1	Short-term toxicity to invertebrates**	≥ 100 tonnes/year	End-points measured in these tests are relevant and applicable for nanomaterials but development of specific test systems/guidelines, with respect to further markers/parameters for toxicity is required (OECD, 2009). Proposed to include short-term ecotoxicity testing at the lowest tonnage level.
9.4.2	Effects on soil micro-organisms	≥ 100 tonnes/year	
9.4.3	Short-term toxicity to plants**	≥ 100 tonnes/year	End-points measured in these tests are relevant and applicable for nanomaterials but development of specific test systems/guidelines, with respect to further markers/parameters for toxicity is required (OECD, 2009). Proposed to include short-term ecotoxicity testing at the lowest tonnage level.
9.4.4	Long-term toxicity testing on invertebrates	≥ 1000 tonnes/year	End-points measured in these tests are relevant and applicable for

9.4.6	Long-term toxicity testing on plants	≥ 1000 tonnes/year	nanomaterials but development of specific test systems/guidelines, with respect to further markers/parameters for toxicity is required (OECD, 2009). Proposed to include long-term ecotoxicity testing at a lower tonnage level.
9.5.1	Long-term toxicity to sediment organisms	≥ 1000 tonnes/year	
9.6.1	Long-term or reproductive toxicity to birds	≥ 1000 tonnes/year	

* In general, monitoring of changes in the physical form and characteristics of nanomaterials during toxicological testing is recommended, as this is instrumental for read-across approaches in the future. ** These parameters (may) need adaptation for nanomaterials (see main text for further details).

2.5 Comparison of drug development and MNM development

The safe by design concept has had a long history of successful deployment in the domain of drug development. In early phase drug development, new chemical entities are screened in parallel for both their efficacy and their potential for toxicity. These screening methods are attractive in that they are fast, cheap, easily reproducible, and that they reduce the number of animals needed for toxicity testing by eliminating animal studies or providing specific targets for further animal or *in vitro* studies. Such an approach might include relevant building blocks for the uptake and development of the safe by design concept for MNMs. In this chapter, 3 questions will be addressed regarding the comparison of drug development and MNM development:

1. What are the similarities and differences in toxicity testing aims between drug and MNM development?

2. What are the critical safety questions in the drug development process? Furthermore, do they apply to the MNM process also?

3. Could the toxicity tests for drug development be applicable for MNMs also?

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1. What are the similarities and differences in toxicity testing aims between drug and MNM development?

Similarities throughout the drug and MNM development can be found in the goals that have to be achieved. Three main goals can be distinguished:

i) one compound (only) has to be developed and industrialised as product for the market,

ii) the compound has to be safe for humans and the environment and

iii) careful consideration and dataset have to be investigated and generated to predict and/or improve the overall safety across the many aspects to consider.

However, how these goals have to be achieved differs between drug and MNM development. Some distinct differences are presented in Table 11 and are divided into starting position, *in vitro* testing, *in vivo* testing and others.

Table 11. Distinct differences between drug development and MNM development

	Drug development	MNM development
Starting position	5000-10000 test compounds	One or few compounds
	Library of similar compounds	Library not available
	Large suite of similar chemical compounds	One or a small suite of similar chemical compounds
	Clear specifications of application (drug for oral use)	Potential for applications
***In vitro* testing**	Huge library of screenings data	Library not available
	Clear criteria for HTS	Criteria for HTS not available
	Screening for severe toxicity	Identifying toxicity (potential)
	Screening for good absorption	
	Information supportive for *in vivo* studies	Partial proof for safety
***In vivo* testing**	Animal studies do not need to be conclusive	Animal studies need to be conclusive
	Human data	No human data
	Enormous amount of toxicity data	Relatively modest amount of toxicity data
Other	Insight into the relevance of test outcomes for business model	Unknown if MNM lead to product(s)

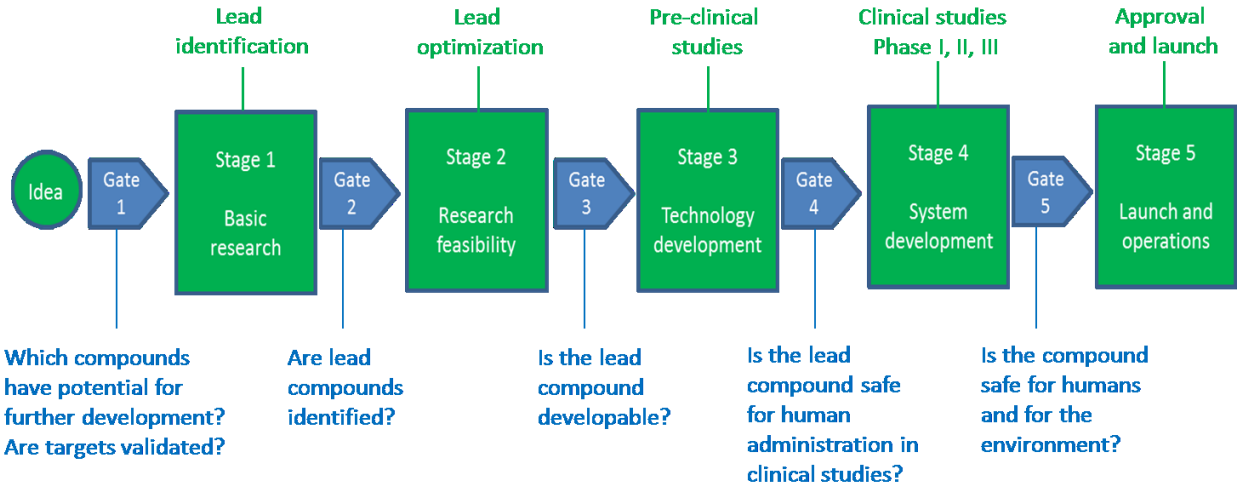
In conclusion, due to the vast amount of information and regulations available for drug development, it is clear what kind of product should be developed.Conversely, this it is not of immediate naturefor the MNMs and it these could have any potential lead to become an industrial products. Both cases have to meet the same common objective set to be "the development of an exploitable lead compound (or MNM). *In vitro* results, during drug development, are i) substantiating the *in vivo* studies, or ii) contribute to unveil mechanism of interactions. For the MNM development, *in vitro* results are predicting toxicity and provide the evidence that guides towards the *in vivo* studies. Thus, *in vitro* results provide different contribution to the development process and therefore start from different requirements, i.e. in drug development they have a guiding function whereas in MNM development they need to be predictive. Furthermore, in many cases limited or no toxicity data in humans (such as clinical trials) is requested for the MNM development, whereas during the drug development, this aspect is a compulsory requirement where to test the chosen compound in both healthy and in target populations. This has implication on the scope of assessing human safety through

different approaches. Finally, an enormous amount of toxicity data is available during drug development, while relatively modest amount of toxicity data is present for MNM development. And further, both are looking for the most complete dataset that could allow for better or faster prediction and/or improvement of the human and environmental safety. It of importance that the implications are immediate since this will allow for a faster translation of products into the market with a higher safety record and profile. However, considerations on how to assess these are still unmet as presented above.

2. What are the critical safety questions in the drug development process? Furthermore, do they apply to the MNM process also?

The five critical questions that need to be addressed during drug development are the questions of the five gates as presented in Figure 8.

Figure 8. Drug development stages and critical safety questions in the innovation model



These questions do not completely fit within the MNM development; however, constitutes a starting point attempt. Here we describe an example of gate questions for MNM, which were presented in the innovation model in Figure 9.

Gate 1

Drug: Which compounds have potential for further development? Are targets validated?

MNM: Which compounds have potential for further development within the intended industrial objectives?

Gate 2

Drug: Are lead compounds identified?

MNM: Does the MNM meet the safety requirements for further development?

Gate 3

Drug: Is the lead compound developable?

MNM: Has the pristine MNM been assessed and declared safe to be explored further?

Gate 4

Drug: Is the lead compound safe for human administration in clinical studies?

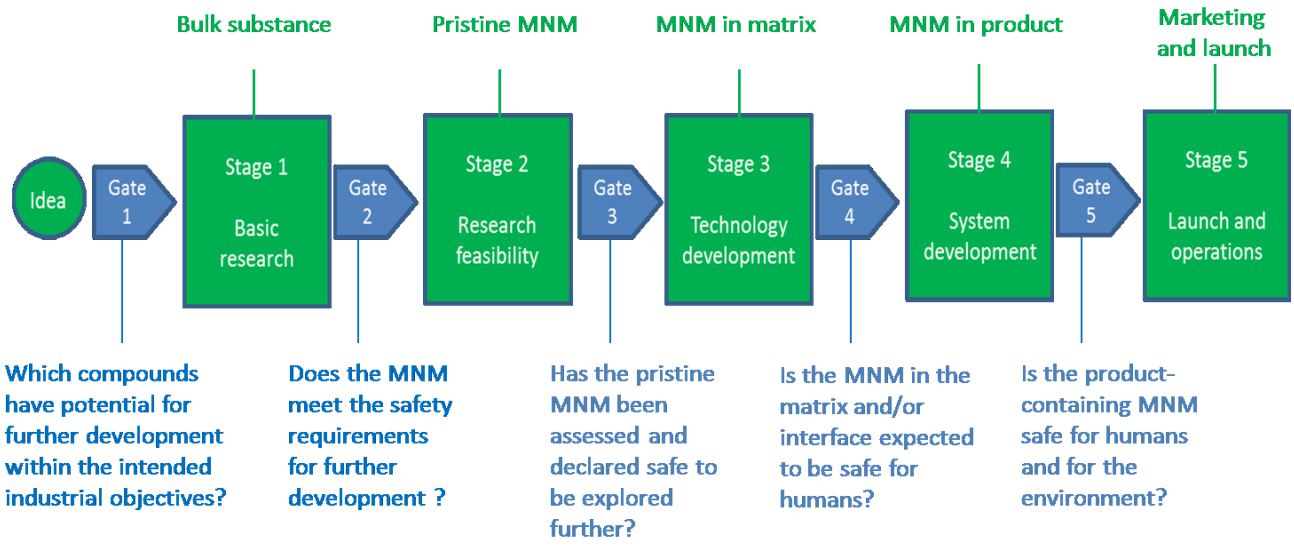
MNM: Is the MNM in the matrix and/or interface expected to be safe for humans?

Gate 5

Drug: Is the compound safe for humans and for the environment?

MNM: Is the product-containing MNM safe for humans and for the environment?

Figure 9. MNM development stages and critical safety questions in the innovation model



In conclusion, comparison between toxicity screening processes have been highlighted in the context of the respective development process and from this it emerged that the basis for the introduction of a safe by design approach within the MNM development is existing and strong. A critical path for development-based on the stage-gate model has also been brought forwards and this has allowed for common consideration and evaluation of the two development processes under comparison. Valuable aspects have been identified which are beneficial to the MNM future innovation process.

3. Are the toxicity tests for drug development applicable for MNMs?

The development of nanomaterials and their increasing use in all sorts of industrial applications and consumer products, challenges the regulating authorities to develop frameworks which can adequately control the potential hazards and risks of these nanomaterials. In Europe, such a framework is the REACH regulation. Since REACH deals with substances, in whatever size, shape or physical state, substances at the nanoscale are also covered by REACH and its provisions apply. This implies that also the safety of nanomaterials to human health and the environment should be ensured under REACH, covering their whole life cycle. However, Pronk et al. (2009) concluded that the European chemicals legislation REACH needs some adjustments to assess and control the risks of nanomaterials.

Text box 6. Concerns on human health toxicity tests within REACH

In general, toxicity testing methods as established for non-nanomaterials are considered suitable for the determination of the effects of MNMs on human health. However, there are a couple of concerns (Rocks et al., 2008; Pronk et al., 2009):

- Mass concentration – Mass concentration (in mg/kg or mg/mL) may not be an appropriate metric for dosage of nanomaterials.
- Appropriate route of exposure – For initial *in vivo* toxicity testing methods normally the oral exposure route is used. However, for testing of MNMs, this may not be sufficient and administration via dermal or inhalation routes is likely to be more applicable. Furthermore, the effect of oral administration of MNMs on gut flora may show toxic effects, which are not investigated and identified during routine toxicity testing (which also counts for bulk materials).
- Duration of tests – Sub-chronic or chronic studies are likely to be the most appropriate to study the toxic effects of MNMs since the duration of human exposure to small amounts of MNMs will be over a longer period of time. Single or short-term exposures are likely to occur with high concentrations of MNMs as a result of accidental release. This point also holds for non-nanomaterials.
- Detection of MNMs – Whereas the potential toxic effects of MNMs will be detectable by using light microscopy, their presence, as single particles or in small aggregates, will not be. Therefore, to show the presence of MNMs within a histological sample it will be necessary to use EM, which may be very laborious and time consuming.
- Distinction and identification of MNMs – As the normal analytical detection methods may not be suitable to detect the presence of nanomaterials within a sample (see above), and EM techniques only show their presence, not their chemical structure, additional techniques such as EDX and XPS should be applied to elucidate the structure. This is essential for the identification of nanomaterials (both manufactured and naturally occurring).
- Systemic effects of toxicity – The most probable scenario is that a nanomaterial, after entering the body, will relocate in the organism and exert a systemic effect at a target site. This cannot be determined by single cell *in vitro* studies and therefore the need for animal experimentation remains until more developed screening tests are available or the relationship between the physicochemical properties of a nanomaterial and its toxic effect can be determined. Again this concern also holds for bulk materials.
- Effect of particulate number – Given the small particle sizes of MNMs and the normal dosimetrics in toxicity studies, there is a distinct possibility that due to the large amount of MNM to be administered (which may no longer be representative for the actual exposure situation), toxic effects induced are a consequence of an overload phenomenon, rather than a consequence of exposure to the MNM itself (or a combination of both).
- Solution or suspension of (nano)material – the distinction between a solution or suspension of a material, whether in nanoform or in bulk form, for use in sample preparation must be considered. However, it is likely that this will only be a problem with long term administration of the test substance as the suspension may precipitate out over time (sediment).
- Use of appropriate solvent – whilst the test nanomaterial may be soluble and stable in an organic solvent, the effects of the solvent on the test system must also be considered. Conversely, the potential of the nanomaterial to interact with the surrounding media (*e.g.* plastic of syringe, cell culture media) must also be considered in the administration of the nanomaterial. This concern also holds for bulk materials.

Text box 7. Concerns on ecotoxicity tests within REACH

The ecological information required under REACH of MNMs was considered in depth by Crane and Handy (2007). The areas where the current ecotoxicological testing methodology was identified as not fit for purpose were:

- Relating macroscale to nanoscale – Current chemicals regulations (including REACH) do not distinguish between the nanoscale and macroscale forms of substances, so ecotoxicity tests performed on the macroscale form may, from a legal point of view, need to be accepted for both macroscale and nanoscale forms by regulatory authorities. This needs to change so that, at the very least, an evidence-based case is presented by manufacturers to show that there is no difference in the hazards of nanoscale and macroscale forms of the same substance. At present macroscale material toxicity cannot be related to nanoscale material toxicity, so currently evidence can only come from (rapid) testing;
- Exposure in test systems – Organisms in ecotoxicity tests should be exposed to nanomaterials in a way that is environmentally relevant. The homogenous dispersion currently recommended in ecotoxicological testing may not reflect this. In the environment nanomaterials may react to their surroundings by agglomeration and aggregation after which precipitation is likely to occur, or they may react with other (naturally occurring) substances that may attach themselves to the surface of nanomaterials;
- Acute to chronic extrapolation – In most environmental risk assessment frameworks chronic toxicity is predicted from acute toxicity data by applying (large) assessment factors. For nanomaterials there is currently not enough empirical data (including data on bioaccumulation potential) to derive such assessment factors;
- Mass concentration is commonly used as a determinant of dose, but other metrics like for instance (combinations of) specific surface area, particle size, zeta potential, and shape might be better suited to quantify adverse effects across nanomaterials.
- Partition coefficient – There are some concerns about whether or not the partition coefficient test works for nanomaterials. This has implications for risk assessment strategies that use the partition coefficient as a trigger for requiring either sediment toxicity tests or bioaccumulation studies.

Since the toxicity tests within REACH are not sufficient for MNMs, toxicity tests used for drug development may be applicable after taking into account the “nano” aspects/properties into the testing protocols. In this deliverable, we presented an overview of toxicity tests used for drug development. However, for now it is not possible to draw conclusions about the applicability for MNMs in general. What we can conclude is that some aspects of drug development may be further developed for screening of risk potentials for MNMs. Based on views developed within RIVM (paper in preparation) and discussion within WP6, the following risk potentials for MNMs are proposed:

- Solubility
- Stability (of coating)
- Accumulation
- Genotoxicity/carcinogenicity
- Inflammation
- Ecotoxicity

When focussing on these risk potentials of MNMs, parameters describing and quantifying such potential have to be defined. Furthermore, there is also a need for the definition of the relationship between the parameters and the risk potentials. Once these parameters are identified and described in detail, then the next step will be the identification of the most satisfactory testing strategy to measure these parameters for qualitative and quantitative assessment. To this aim, the experiences from drug development could provide a great valuable and lesson learned experience.

3 Evaluation and conclusions

The Full Assembly deems this deliverable to be fulfilled satisfactory/not satisfactory.

In the latter case, please make a statement about the state of affairs regarding impact of failure, and contingency plan.

4 Deviations from the work plan

There is one deviation from the workplan. The Safe by Design concept was not intended to be discussed in this deliverable. However, during the NANoREG project, RIVM has developed new ideas on a safe innovations approach that includes the Safe by Design concept. It was thought that this Safe by Design approach could be relevant and useful for the comparison of drug development and MNM development. Therefore, the WP-leader and task-leader have decided, in consultation with WP-partners, to include the Safe by Design approach in this deliverable.

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