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# SYSTEMS TOXICOLOGY APPROACH IN NANOSAFETY:

# FROM *IN VIVO* TOWARDS *IN VITRO* TESTING

**Pia Kinaret**

ACADEMIC DISSERTATION

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*What the mind doesn't understand, it worships or fears.*

*Alice Walker*

# ABSTRACT

Nanotechnology and engineered nanomaterials (ENM) are providing outstanding innovations in several fields of science and technology, spanning from medicine to aeronautics. However, some ENM are known to be harmful to humans and the environment, thus, their toxic potential need to be thoroughly tested.

The production of ENM is drastically increasing, making traditional toxicity testing impractical and in some cases impossible. The classical hazard assessment involves extensive animal exposures, which are not applicable to vast growing number of novel nanomaterials. Their unique properties and behaviour in biological entities are differing from their bulk sized counterparts, making predictions of the effects difficult. Thus, new efficient and rapid methods need to be developed for nanomaterial toxicity testing and hazard assessment.

The main ENM exposure route to humans is through airways. Studying the effects of nanoparticles and their toxic potential on the airways is hence necessary for creating a comprehensive understanding of the ENM-induced phenotypic, cellular and molecular changes. For this, animal models are widely recognized as the best method to mimic the ENM exposures to humans. Inhalation exposure to murine models is considered as the state of the art method for studying the pulmonary responses. This expensive and laborious method is not practical for testing all nanomaterials with all the relevant doses. In another airway exposure method, the oropharyngeal aspiration, the ENM are introduced to the airways of test animals as a liquid dispersion under anesthesia. The aspiration method is debated, since the anesthesia and the dispersion might cause additional effects and responses.

In the first part of this thesis, these two different airway exposure methods were compared, aiming at understanding whether the easier and faster oropharyngeal aspiration method could substitute the time-consuming and expensive whole-body inhalation method. The conjecture was indeed valid, as both methods showed similar outcomes at cellular and molecular level in response to rigid multi-walled carbon nanotubes after four-day exposure.

Since the ongoing effort in reducing laboratory animal testing in hazard assessment as well as for faster and simpler testing, methods for replacing animals are being examined also by *in vitro* exposures. Cell exposures are even more argued, since they do not resemble the comprehensive responses of an animal and even less of the human organism, where different cell types are interacting and communicating in very intricated manner. Novel computational methods are being developed to mine the large exposure data

sets for finding relevant features, that would explain and predict the human responses to ENM from *in vitro* and *in vivo* exposures.
In the second part of the thesis, the possibility to interpret the complicated pulmonary responses by examining the transcriptional patterns of human macrophages and mouse lungs when exposed to carbon nanomaterials (CNM) were investigated. Gene co-expression networks revealed specific molecular patterns related to distinct properties of the CNM, namely aspect ratio, length, diameter and surface area, but which were common between *in vitro* and *in vivo*.

These results aid the establishment of systems toxicology approaches in ENM hazard assessment and the shift from the classical, animal-based hazard assessment towards predictive models, ultimately supporting the development of new "safe-by-design" nanomaterials (safe design, safe production, and safe use).

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Helsinki 2017

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# LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I **Kinaret P**, Ilves M, Fortino V, Rydman E, Karisola P, Lähde A, Koivisto J, Jokiniemi J, Wolff H, Savolainen K, Greco D, Alenius H, 2017. Inhalation and Oropharyngeal Aspiration Exposure to Rod-Like Carbon Nanotubes Induce Similar Airway Inflammation and Biological Responses in Mouse Lungs. *ACS Nano*, 11:291-303.

II **Kinaret P**, Marwah V, Fortino V, Ilves M, Wolff H, Ruokolainen L, Auvinen P, Savolainen K, Alenius H, Greco D, 2017. Network Analysis Reveals Similar Transcriptomic Responses To Intrinsic Properties Of Carbon Nanomaterials In Vitro And In Vivo. *ACS Nano*, 11:3786-3796.

The publications are referred to in the text by their roman numerals.

# ABBREVIATIONS

| | |
|---|---|
| AOP | Adverse outcome pathway |
| ATP | Adenosine triphosphate |
| BAL | Bronchoalveolar lavage |
| BN | Biological network |
| BSA | Bovine serum albumin |
| CCL | Chemokine C-C motif ligand |
| CNM | Carbon nanomaterials |
| DAMP | Damage-associated molecular pattern |
| DNA | Deoxyribonucleic acid |
| ECM | Extracellular matrix |
| ENM | Engineered nanomaterials |
| EU | European Union |
| FBS | Fetal bovine serum |
| GO | Gene ontology |
| HARN | High aspect ratio nanomaterials |
| HPF | High-power field |
| H&E | Hematoxylin and eosin (stain) |
| IgE | Immunoglobulin E |
| IL | Interleukin |
| *In silico* | Performed on a computer |
| *In vitro* | Performed outside of living organism |
| *In vivo* | Performed within the living organism |
| KEGG | Kyoto Encyclopedia of Genes and Genomes |
| KET | Key enabling technologies |
| *Ex vivo* | performed in or on tissue/organ, outside the living organism |
| MoA | Mode of action |
| MOA | Mechanism of action |
| MWCNT | Multi-walled carbon nanotube |
| NAMP | Nanomaterial-associated molecular pattern |
| NASA | National aeronautics and space administration (U.S.) |
| NOAEL | No-observed-adverse-effect-level |
| NRC | National Research Council (U.S.) |
| OECD | Organisation for Economic Cooperation and Development |
| PAMP | Pathogen-associated molecular patterns |
| PAS | Periodic acid-Shiff (stain) |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| PEG | Polyethylene glycol |
| PMA | phorbol 12-myristate 13-acetate |
| PoT | Pathways of Toxicity |
| RA | Risk assessment |

| | |
|---|---|
| rCNT | rigid multi-walled carbon nanotubes (Mitsui-7) |
| REACH | Registration, Evaluation, Authorization and Restriction of Chemicals |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| SEM | Scanning electron microscope |
| SWCNT | Single-walled carbon nanotube |
| tCNT | Tangled multi-walled carbon nanotubes (Cheaptube) |
| TEM | Transmission electron microscope |
| THP-1 | Human monocytic cell line |
| Th1 | T helper cell type 1 |
| Th2 | T helper cell type 2 |
| TMOA | Transcriptional mechanism of action |
| TOX21 | Toxicology in 21st Century |
| RT-qPCR | Real-time/reverse transcriptase quantitative polymerase chain reaction |
| QSAR | Quantitative structure-activity relationship |
| 3R | Reduction, refinement and replacement of animal testing (3R principle) |

# 1 INTRODUCTION

Consider the dot at the end of this sentence. Imagine dividing that 0.4-millimeter ink mark *hundred thousand* times. The particles in that size range, not visible to your eyes and quite difficult to even imagine, might be capable of revolutionize human lives.

One of the major breakthroughs in medicine was achieved in year 1717, when Lady Mary Wortley Montagu introduced smallpox inoculation to the Western medicine. This was followed 80 years later by the successful vaccination with cowpox by Edward Jenner. Nowadays, vaccinations save 2 to 3 million lives worldwide every year (Shin, Shin, & Ki, 2008; World Health Organization. Global Programme for Vaccines, Immunization, UNICEF, 1996). Nanomedicine, in which nano-sized particles carrying a drug molecule, target a specific site of the human body, for example an inflammatory site or a cancerous tissue, might revolutionize the global health once more (Pautler & Brenner, 2010).

Engineered nanomaterials (ENM) have enormous potential in a variety of industrial applications. For this, the European Union (EU) has elevated nanotechnology as one of the Key Enabling Technologies (KET) in Europe's industrial policy in 21st century, together with micro and nanoelectronics, industrial biotechnology, advanced materials, photonics, and advanced manufacturing technologies. These innovative technologies, are facilitating profound changes to the society, culture and several industrial sectors (Savolainen *et al.*, 2013). In fact, this ground-breaking technology is stimulating economic growth, creating new jobs, preserving nature and ultimately making our everyday lives easier in many ways. Nanotechnology has taken several leaps forward in the 21st century, when ENM started to emerge in consumer products such as food, sports equipment, cosmetics and electronics. An inventory from year 2013 listed over 1,800 ENM-based consumer products from more than 600 companies in 32 countries. The highest number of ENM-products (42% of the total), was from the health and fitness category (Vance *et al.*, 2015). In year 2016, the majority of the registered nano-products belonged to the category of "personal care" and "clothing", followed by "sporting goods" and "cleaning" (Hansen *et al.*, 2016). Currently, 3,005 products containing nanomaterials are registered and available on the European market (**Figure 1**) (The Nanodatabase: http://nanodb.dk).

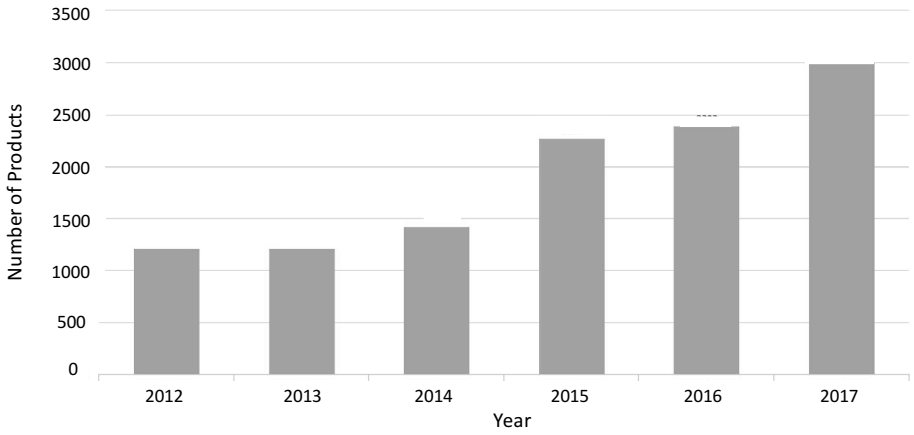


**Figure 1** Registered products containing engineered nanomaterials in EU from 2012 until June 2017 (downloaded from http://nanodb.dk).

Nanomaterial applications are virtually endless, as are their different shapes, sizes and structures. For their very reactive nature, nanomaterials cause also concerns, and there is increasing need in investigating their toxic potential. Nano-sized particles can, in fact, also represent unrecognized threats, as the same particles able to improve health and protect the environment, can also cause harm if not carefully controlled. What makes this matter more difficult, is that we don't exactly know how to distinguish the good from the bad. Even more philosophically thinking, the good can also be bad, depending on the viewpoint angle.

All new substances brought onto the market should be safe to human and the environment and a plethora of toxicity tests are developed to assess safety risk. Traditional toxicity tests specifically address the safety of chemical substances, and thus, might not be applicable to ENM, because of their unique features. For this reason, we need novel approaches to thoroughly understand ENM intrinsic characteristics, as well as their behavior when in contact with biological entities and the environment.

The main aim of toxicity testing is to generate adequate information on a substance (*e.g.* ENM) hazardous effects and their underlying mechanisms. Classical toxicity tests are primarily animal-based, laborious and expensive. They typically require large amount of the test substance and therefore are not practical for nano-scale particles, while there is a lack of scalable and robust methods for large scale testing. Due to the constantly growing number of new nano-particles developed, novel methods and testing strategies that are able to predict ENM safety in human are urgently needed. Human cell based cultures and tissue models, together with predictive computational

models, could be a more appropriate choice. Systems toxicology aims at shifting the emphasis from the traditional (animal and, histopathology based) approaches to predictive (mechanistic based) risk assessment. Systematically collecting and integrating information from *in vivo* and *in vitro* exposures as well as the use of novel computational methods, ensures more robust and faster risk assessment.

With the aid of novel computational methods, the possible danger could be properly estimated from the properties of the chemicals and particles, as well as further enabling the design and production of merely non-toxic materials and products. With predictive systems toxicology approaches, also ethically questioned and massive animal experimentations could be decreased, facilitating the 3R principles (replacement, reduction, refinement) of animal testing.

Since one of the major route for nanomaterial exposure to humans is *via* the airways, inhalation exposure to animal models is used as a state of the art method in ENM toxicity testing. In this methodology, the animal is breathing aerosolized nanomaterial. The effects of the exposure are then studied at the level of responding organs, tissue, cells and molecules, aiming at understanding the changes and interactions between biological entities and ENM. This expensive and laborious method is not scalable to test all nanomaterials at all needed doses for complete understanding of the possible health risk. It also requires costly equipment and expertise from several fields, such as material physics, biology, biotechnology and animal housing and handling. In addition, inhalation method is excessively time consuming to allow a rapid assessment of toxicity for larger number of different nanomaterials. Oropharyngeal aspiration is an alternative method, in which the material is introduced under anesthesia into the airways as a liquid dispersion. However, the effectiveness of this procedure is debated, for the anesthesia and the saline solution aspirated to lungs might possibly mask the responses to ENM. The ongoing effort in reducing animal testing, is at the same time promoting the development of *in vitro* cellular methods. The value of cell-based testing is also argued, since they may not resemble the adequate responses of a living organism, in which different cell types are interacting in a complex manner.

In this thesis, first, the possibility to replace ENM inhalation exposure method with simpler and cheaper aspiration exposure method was investigated in mice. Secondly, the *in vitro* and *in vivo* testing methods were investigated, in order to provide a new non-animal testing strategy to predict *in vivo* effects of ENM from *in vitro* experiments. Lastly, the capability of transcriptomics based network analysis as part of ENM´s hazard assessment was evaluated. This work contributes to our understanding of the adverse responses of multicellular organisms exposed to nanomaterials as well as to the development of innovative and more efficient safety testing strategies.

# 2 NANOTECHNOLOGY

In order to understand the concept "nanotechnology", we need to scrutinize the nomenclature behind it. *Nânos,* is an Ancient Greek term for "dwarf" (Latin: *Nanus*), used in European folklores already before 13th century, to describe a small creature having supernatural powers such as healing and wisdom.

To grasp the modern terminology for nano-sized material, in other words a particle with at least one dimension less than 100 nanometres (nm), we must place it into a concept. As an example, the smallest particle visible to human eye is approximately 40 micrometres (40,000 nm). That is the size of some bacteria or plant cells. *Variola*, a large smallpox virus instead, ranging from 200 nm to 400 nm, targets eukaryotic animal cells, which vary from 10 to 30 micrometres. One sheet in the book you are holding, is around 100,000 nanometres thick. **Figure 2** represents common objects in nanometre scale.

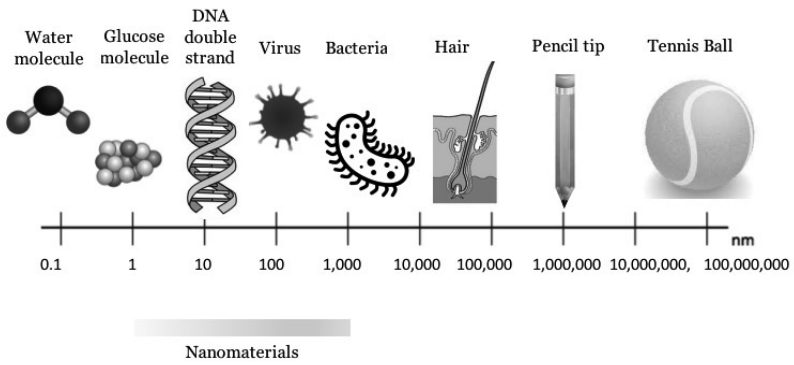


**Figure 2** Examples of different objects on nanoscale. Adapted from Savolainen *et al.* (Savolainen *et al.*, 2013).

Nanos have always been around us, actually before humans and animals even populated the earth. Essentially, nanos are not bound to our planet at all, but are particles of the universe. What associates them to our planet and us, is the word "technology", which can be understood as "deliberately created", "modified" or "processed", aiming at providing new products, knowledge or services benefiting mankind. Miniaturization enables us to manipulate molecules and atoms, as envisioned by the physicist Richard Feynman already in 1959 (Feynman, 1960). Intentionally produced nanomaterials, are referred to engineered nanomaterials (ENM), and can be developed from a variety of chemical elements such as gold, silver, titanium, cadmium, carbon, or from artificial and synthetic materials. Shapes of ENM

range from tubes, sheets, spheroids, polyhedrons, cubes, cones and to everything in between.

Why do we want to create and produce particles so small? For the same reason, why our ancestors were fascinated about the mystical powers of *Nanus*. The small size comes with great benefits. Nowadays the supernatural powers of ENM are not so mystic, but still somewhat mysterious and not thoroughly understood.

Several industrial and medical fields focus on nanotechnology-based applications. Medical technology and healthcare are aiming at harnessing ENM to detect and fight diseases, to kill microorganisms such as bacteria and viruses, as well as to repair and regenerate damaged tissues (Pautler & Brenner, 2010). The USA National Aeronautics and Space Administration (NASA), along with several industrial sectors, are examining numerous possibilities for utilizing carbon nanostructures in building crafts, vessels, cars and other products with enormous strength and flexibility at the same time. Simultaneously, food industry is examining, and already exploiting nanomaterials in food packaging, *e.g.* exploiting nano-silver's beneficial antimicrobial activity. Nanomaterials in food can make an impact on taste and texture, as well as to help distributing the nutrients and vitamins to us in more efficient manner.

The above-mentioned inventions unveil the huge impact that ENM might have to us and to the environment. If we are able to harness and master nanomaterials and their properties, we indeed have great possibility to make radical changes in fields such as electronics, medicine, aeronautics, food industry, *etc.* But, before we can fully benefit from the use of ENM, we need to thoroughly understand their characteristics, behaviour and possible effects to humans and the environment.

## 2.1 CARBON NANOMATERIALS

Carbon-based nanomaterials (CNM) possess numerous beneficial properties such as remarkable strength, elasticity, electrical and thermal conductivity and chemical inertness. CNM consist of covalently bonded carbon atoms, arranged in a honeycomb structure. The arrangement of the atoms and the strong bonds between them, can make CNM much stronger than steel. CNM are highly beneficial in numerous applications from sensors to energy storage, drug delivery and diagnostics. Fullerene, a spherical carbon nanomaterial, named as Buckminsterfullerene (Buckyball) was the first nanomaterial discovered by chemists Harry Kroto, Robert Curl and Richard Smalley in year 1985 (Baggott, 1996; Kroto, 2010). The potential benefits of this new particle were quickly understood, and the finders were awarded with the Nobel prize for chemistry in 1996. The best-known allotrope of carbon is diamond, which can be also miniaturized to nano-scale. Because of

the chemical inertness and biocompatibility, nanodiamonds are currently investigated as possible drug carriers (Ho, Wang, & Chow, 2015; Lim *et al.*, 2016).

Another Nobel prize in year 2010, was awarded in physics to Andre Geim and Konstantin Novoselov, from the revolutionary experiments on graphene (The Royal Swedish Academy of Sciences). Graphene, synthesized from the graphite mineral, is a sheet of carbon atoms, as thin as one carbon atom itself. These graphene sheets can be remodified as hollow tubes, *i.e.* single-walled carbon nanotubes (SWCNT), as well as double or multi-walled carbon nanotubes (MWCNT), consisting of multiple layers of graphene sheets coiled around each other.

In addition to the atomic conformation, carbon nanomaterials can vary in size, shape, charge, surface area and reactivity. All the aforementioned properties affect their biological identity, making it difficult to study all the possible effects and interactions with biological systems. For this reason, along the numerous beneficial properties and applications of carbon nanomaterials, also concerns about their safety has been raised, while the beneficial properties might be also harmful to the human health and the environment. Therefore, tests mimicking human exposure and responses are urgently needed.

## 2.2 EXPOSURE TO NANOMATERIALS

While ENM are highly beneficial in many applications and industrial fields, also concerns have risen for their potential to react in unpredictable ways when brought in contact with biological entities. Several ENM characteristics are known to affect the toxic potential of nanomaterials. The toxicity depends from both synthetic and biological identity of the ENM. The intrinsic identity includes chemical composition and crystalline structure, as well as surface coating, porosity, crystallinity, shape, surface area, aspect ratio and size (Hristozov, Gottardo, Critto, & Marcomini, 2012). The biological identity on the other hand, depends on the colloidal forces and the dynamics between the material characteristics and the encountered environment. For this, thorough characterization of the ENM in different biological environments should be carried out before drawing conclusions about their toxic potential.

Many of us are getting symptoms such as coughing and irritation in response to ultrafine, airborne particles found in dust and pollen. Similarly, as dust and diesel exhausts, which also contain nano-sized particles, ENM move effortlessly in the surroundings. The minor effect from gravity enables ENM to float in the air, and therefore allow an easy access to airways by inhalation. Likewise, also unintentional skin or eye exposure to ENM is possible.

Because of their extremely small size, some particles might be able to penetrate the biological barriers of the human body. Cells of the immune

system are normally recognizing, destroying and clearing the foreign particles in efficient manner. It is also well established, that some nanomaterials, especially long and rigid, fibrous structures are biopersistent, causing impairment of immune cells, chronic inflammation and tissue damage (Oberdörster *et al.*, 2005a). At the same time, since the minuscule size of ENM, immune cells that would usually process and remove the foreign particles, might not recognize these small intruders, letting them to travel and possibly relocate to organs such as spleen, liver or kidneys. If the particle is internalized by the cell, uncontrolled intracellular trafficking and responses might lead to interference of normal cellular processes.

Currently, the highest risk of ENM exposure is in occupational settings, for workers producing the ENM in manufacturing processes, mainly by inhaling or through dermal exposure. As the level of ENM-based consumer products keeps increasing, the risk of getting exposed to ENM is affecting all populations and the environment at global scale (Oberdörster *et al.*, 2005a).

ENM might possess higher toxic potential than their bulk-sized counterparts. While the size of the particle decreases, its surface to volume ratio increases, as more atoms are getting into contact with the surroundings. Thus, the surface area greatly affects to the reactivity of ENM, and is considered as an important factor when examining ENM effects to biological systems. As mentioned earlier, a known influence of high length to diameter ratio of ENM, referred to as the aspect ratio, especially in relation to asbestos fibres and carbon nanotubes, has also an impact on cellular responses.

ENM might behave differently depending on the surroundings they encounter. When the particle travels through airways for instance, the molecules present in biological fluids rapidly stick to the ENM, forming biological corona on the surface of the particle. This bio-corona affects the ENM behaviour and properties. The attached surface molecules might cover the ENM surface, and in this way, alter the particle reactivity and behaviour. ENM might also be intentionally coated with known molecular structures. Functionalization of ENM is currently exploited in medical purposes, where ENM could be harnessed to carry drugs to specific sites of the body, or trigger and recruit cells and molecules of the immune system. Medical applications of ENM are still limited, since it is not known whether ENM are completely cleared from the body after it has completed its task, or translocated to another site on the body, causing harm instead of benefits. The same difficulty applies to ENM that are unintentionally internalized due to exposure. Researchers have found evidence of ENM biodistribution to liver, kidneys, heart and even brain after exposure.

How ENM behave after exposure depends on several factors, which need to be considered when studying ENM exposure. The Organisation of Economic Cooperation and Development (OECD) has addressed the importance of characterizing physical-chemical parameters of ENM that

need to be taken into account in nanomaterial safety regulation (Rasmussen *et al.*, 2016). Thus, harmonized methods and particle characterization, the use of known reference materials and controlled conditions for measurements are of great importance when ensuring ENM safety. This requires global, shared legislation and strategy for ENM production, manufacturing and safety testing. Unified risk and hazard assessment of ENM, as well as controlled production and distribution need to be evaluated. Securing environment and safety of the vulnerable populations, needs to be considered as well. Before complete understanding of the possible harmful characteristics of ENM, we are unable to respond to the societal needs for safe ENM, or to develop new ENM-based riskless innovations. It is important to realize, that the current ENM on the market are mainly passive nanostructures, – so called "first generation" materials, whereas second, third and fourth generation of ENM will likely include active structures, nanosystems, and nanorobotics (Savolainen *et al.*, 2013). Next generation ENM will open many great possibilities, but also create even more complicated risk and safety assessment, requiring development of novel toxicity testing approaches and predictive methods.

Nanotechnology has a great promise for the future. To ensure the innovativeness as well as safe development and handling of ENM, both sides of the double-edged sword need to be thoroughly understood. Detailed engineering of the future ENM, would allow production of safe nanomaterials and facilitate the revolution of new functional and safe ENM.

# 3 NANO-BIO INTERACTIONS

The human immune system has evolved to protect us from foreign threats and invaders, rapidly responding to recognized danger. If the intruder cannot be cleared from the body in an effective manner, the immune system might get over-activated, causing damage rather than protection. The unique properties of ENM, enable them to interact with biological entities in numerous ways. Because of the minuscule size of ENM, the interactions with biological systems might differ from the interactions of their bulk-sized counterparts. Reactiveness of ENM and their degree of interaction depends from several factors, including their physical and chemical properties and agglomeration status, as well as external factors such as the encountered cell population and the surrounding environment.

Distinct types of ENM and different exposure routes trigger different types of immune reactions. The main routes of ENM exposure to humans are through the airways (respiratory tract), skin (dermal contact), oral (gastrointestinal tract), and intravenous exposures (injections) (Oberdörster, Oberdörster, & Oberdörster, 2005b). Exposure is likely to happen in industrial environment, during production and handling of ENM, but also in everyday life, when consumers are exposed to a variety of nano-products already available on the market. New types of interaction might emerge through nanomedicine, when the material is carefully designed to avoid the immune system, and thus is able to accumulate in our body. ENM can also relocate inside the body after the exposure, causing adverse effects not only in the primarily exposed tissues, but also in other organs such as spleen, liver, heart, kidneys, bone marrow or brain. In this thesis, the focus is on airway exposure, and thus ENM interactions are considered through short-time exposures and mediated by the innate and adaptive immune system of the airways.

## 3.1 BIO-CORONA FORMATION

When a nanomaterial encounters a biological surface, such as the lung tissue and the mucus layer covering the cells on airways, it is rapidly surrounded and covered with biological molecules, such as lipids, carbohydrates and proteins. This biomolecule coating called bio-corona, might have an important role in ENM behaviour in biological environment (Mahmoudi *et al.*, 2011). The formation of the corona depends on the chemistry, charge and reactivity of the ENM itself, as well as from the surrounding biomolecules (Sengupta *et al.*, 2015). While the dynamics of its formation are not well understood yet, bio-corona is known to have an important effect on ENM recognition by the cells of the immune system. In addition to ENM size and

shape, biodistribution and biological interactions with host cells are highly dependent on the molecules on the surface. Bio-corona has an impact on ENM reactivity and accumulation, which might further lead to possible harmful or toxic effects. The exact mechanisms of how the corona is formed, how strong is the impact on ENM reactivity, and which physical and/or chemical properties explain the corona formation remain still unknown, complicating the prediction of ENM behaviour inside the human body.

Intentionally coated ENM instead, are exploited *e.g.* in nanomedicine, to functionalize ENM in several beneficial ways. Deliberate coating is applied to make ENM non-reactive, or unrecognizable by the immune cells. Functionalized ENM can avoid destructive cells of the immune system, and thus travel extended period of time inside the human body. Coating might also protect the nanomaterial from degrading enzymes, enabling persistency inside the biological entity. On the other hand, the functionalization might also be applied to trigger the immune system to target more efficiently against tenacious diseases (Bhattacharya *et al.*, 2016).

## 3.2 NANOMATERIAL TOXICITY

The toxic potential of ENM is mediated by combinations of ENM properties, such as length, diameter or surface area (**Figure 3**), as well as their chemical composition, bio-corona and charge.

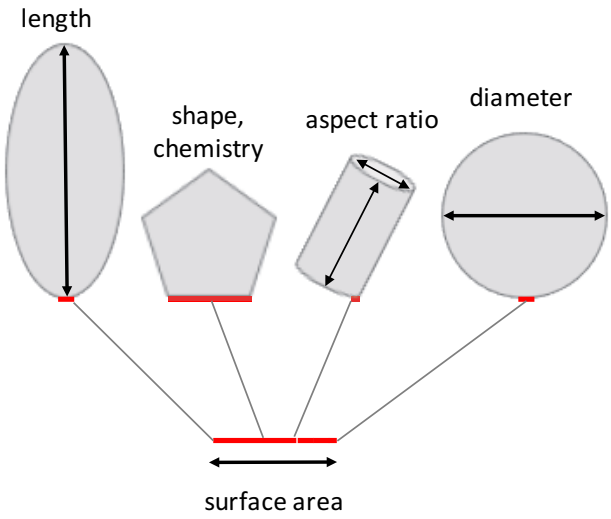


**Figure 3** Geometrical properties of ENM.

To date, many exposure studies have described the toxic effects in response to properties of different ENM. Reasonings about the different toxic

mechanisms of distinct nanoparticles have been also postulated: ion release and dissolution by metal and metal oxide nanoparticles causing *e.g.* cell membrane integrity, and bioaccumulation and biopersistency of long carbon nanotubes (Nel *et al.*, 2013; Woźniak *et al.*, 2017). Especially long and rigid multi-walled carbon nanotubes (rCNT), with high aspect ratio are known to cause severe inflammatory responses in test animals and cell models (Palomäki *et al.*, 2011; Poulsen *et al.*, 2015; Rydman *et al.*, 2014). Nevertheless, the tested exposure scenarios cannot be adequately compared, since the varying test doses, end-points, exposure set-ups and lack of standardized testing strategies in different institutes and laboratories.

ENM toxicity is likely to occur through direct interaction with extracellular matrix and membranes, or through the cells of the immune system. Even though ENM would be completely internalized by professional phagocytes of the immune system, they might not be easily degraded by these cells. Failure to efficiently degrade internalized nanoparticles might, in turn, lead to imbalance in the mitochondrial respiration and cellular metabolism, leading to inflammation, increase in reactive oxygen species (ROS), or fibrosis (Farrera & Fadeel, 2015). Oxidative stress can severely damage the tissue, inducing excessive cell death (David, Owen, & Liptrott, 2016). The toxic potential of ENM is also affected by the dose and time of exposure. However, exposure studies focusing on similar ENM are revealing controversial results: the ENM effects might differ drastically when the exposure route, dose or exposure media are different, or when distinct end points and exposure methods are used. For this, also comparisons of toxic effects between species and exposure methods should be addressed. The variety of fluctuating properties and interactions, makes the study of the ENM interactions and potential harmful effects extremely difficult. In addition, it has also been shown, that ENM might escape the immune system, cross the biological barriers, and thus relocate to other parts of the body, causing new types of interactions with biological entities. Long, carbon nanomaterials for example, are known to be extremely biopersistent, which might promote the establishment of toxic effects, such as local inflammation and granuloma formation (Jacobsen *et al.*, 2016).

### 3.2.1 CYTOTOXICITY

As stated above, chemical and physical characteristics of the ENM are defining how cells are responding to foreign threat. In case of a lethal exposure, for instance, the cell will follow one of the pathways of death: apoptosis, necrosis or “self eating” mechanism namely autophagy (De Stefano, Carnuccio, & Maiuri, 2012).

Apoptosis is the programmed death of a cell, which helps to remove infected or abnormal cells, thus maintaining cellular homeostasis. The bone marrow produces millions of new neutrophils, monocytes and red blood cells every day. To keep balance in the body, equal amount of short lived

leukocytes need to consequently undergo programmed cell death. Apoptosis is, thus, having an important role in the functions of the immune system (Opferman, 2008). In addition to normal maintenance by extrinsic signals, apoptosis can also be induced by distinct stress conditions such as DNA damage or excessive ROS production (De Stefano *et al.*, 2012; Galluzzi *et al.*, 2012).

Necrosis, on the other hand, is usually a sign of major trauma, leading to uncontrolled cell death, inflammation and injury (Mattson & Bazan, 2012). Several stimuli can induce necrosis, including death signals, lack of oxygen or mechanical disruption of the cell membranes. Necrosis is believed to be triggered by exposure to certain ENM. Especially long and rigid carbon nanotubes (rCNT) can cause mechanistic disruption of the cellular membranes as well as granuloma formation by fusion of macrophages. It is hypothesized that in the centre of the granuloma, cells die by necrosis, because of the excessive amount of cytotoxic signals and the lack of oxygen (Murphy, Travers, Walport, 2011). Whether ENM are able to initiate classical necrosis is still debated, since the lack of discriminative biomarkers between forms of cell deaths (De Stefano *et al.*, 2012).

Autophagy maintains the cellular balance by recycling intracellular organelles and molecules (Fan & Zong, 2013). Apoptosis and autophagy are interconnected, able to trigger cell death. Both mechanisms of are often detected after nanomaterial exposure, for example with positively charged dendrimers, gold, iron oxide and silica nanoparticles (Gustafson, Holt-Casper, Grainger, & Ghandehari, 2015; Peynshaert *et al.*, 2014).

Sub-lethal exposure can also lead to decrease in normal cell proliferation and cell growth. Various *in vitro* and *in vivo* studies have evidenced ENM cytotoxicity through uncontrolled inflammation, extracellular matrix (ECM) remodelling and release of reactive oxygen species (ROS) (Khalili Fard, Jafari, & Eghbal, 2015; Pacurari, Lowe, Tchounwou, & Kafoury, 2016). Harmful properties of ENM often arise from their surface reactivity, which is known to negatively impact on the homeostasis of the cellular membranes, causing impairment of membrane integrity (Corbo *et al.*, 2016). ENM surface properties also depend on the particle agglomeration status, altering directly their surface to volume ratio, which in turn, affects to the particle reactivity. Molecules attached to the surface of the ENM, *i.e.* bio-corona, are another important feature affecting to the ENM-cell interaction and possible cytotoxic effects.

### 3.2.2 GENOTOXICITY

When a chemical or substance is capable of altering the genetic information of the cell, it is considered genotoxic. The interaction between toxicant and the genetic material can occur through direct or indirect contact, leading to DNA damage and/or mutations. Cells are able to repair genetic damages to some extent by several DNA repair mechanisms, but exposure to toxic agent

might also result in chromosomal changes, gene mutations, cancer formation or cell death (Christmann & Kaina, 2013).

ENM potential to cause genetic alterations is recognized, but the exact mechanisms causing genotoxicity are not sufficiently clarified. DNA damage can occur through physical interaction between ENM and DNA. ENM might also trigger programmed cell death or increase in production of reactive oxygen species. (ROS) (Ali *et al.*, 2016). ROS in turn affects to the signalling pathways of a cell: Peroxidising the cellular lipids and membranes, altering proteins, disrupting DNA, and modifying transcriptional cascades. It is documented that at least carbon nanomaterials, metal oxides and silica nanomaterials might be able to cause mutations, DNA destruction and imbalance in chromosome separation during cell division by multiple distinct mechanisms (Golbamaki *et al.*, 2015). Even though DNA damage has been widely investigated, only one material, rCNT (rigid MWCNT, Mitsui-7) is known to cause carcinogenicity by mesothelioma formation (Toyokuni, 2013).

### 3.2.3 IMMUNOTOXICITY

Changes in the balance of the immune system may lead to serious consequences, such as autoimmunity diseases, impaired protection towards pathogens, ineffective removal of cancerous cells, hypersensitivity, or damage to tissues and organs (Wang, Reece, & Brown, 2013). Exposure to nanomaterials can also exacerbate immunotoxicological responses through improper cellular activation. Particularly, ENM size has been suggested as a key element in eliciting unwanted immune responses. For example, cells of the innate immune system are struggling to remove long and thin, fibre-like ENM. Innate immune cells, such as the macrophages, are not able to fully engulf bigger particles than the cell itself, triggering a phenomenon known as frustrated phagocytosis. In this situation, macrophages tend to fuse to form giant cells, secreting an excess of pro-inflammatory mediators. The imbalance in immune system might, in turn, facilitate granuloma formation, chronic inflammation and even tissue destruction (Donaldson, Murphy, Duffin, & Poland, 2010). In addition to size and shape, charge and hydrophobicity of ENM have also been associated to excessive inflammatory responses. Indeed, cationic particles are able to activate the immune system more than the anionic or neutral particles (Fromen *et al.*, 2016; 2015). Moreover, hydrophobicity of the ENM, is also known to elicit innate immune responses (Shima, Akagi, & Akashi, 2015). Numerous properties of ENM are identified to cause drastic responses in different organisms. Nevertheless, we still lack a thorough understanding of all the aspects affecting to the biological functions and produced signals, which further influence to the recognition and activated immune responses.

## 3.3 INNATE IMMUNE SYSTEM OF THE LUNGS

Alveolar membrane is the largest surface in human body having a close connection to outside environment, as well as to blood circulation (Martin & Frevert, 2005). Effective oxygen uptake, makes airways also extremely vulnerable to threats such as bacteria, viruses and other foreign particles. Inhaling of ENM is one of the major exposure threats in occupational settings. However, due to rapid development of novel ENM, they might become a risk factor in other environments as well.

Lungs constantly encounter foreign, small particles such as pollution, microbes and dust. For this reason, immune system is constantly alert, with several types of immune cells permanently residing in the healthy respiratory tissues. When foreign particles are inhaled, several mechanisms attempt to clear the particles from the airways. First, particles encounter enzyme-rich mucus layer covering the delicate epithelial surface of the lung tissue. The mucin-rich layer is helping to keep the particles from reaching the epithelial cell lining and the alveolar regions of the airways. Together with mucus secretion and constant movement of cilia, the foreign particles are removed through mucociliary clearance by transferring the particles towards the pharynx, from where ENM are coughed out, or engulfed by digestive system (Jacobsen *et al.*, 2016). If the particle cannot be transported and coughed out, it will encounter the first scavenger cells of the innate immune, residing in the lung surroundings: dendritic cells and tissue resident macrophages (Wang *et al.*, 2013). These professional phagocytes engulf the particles and rapidly signal to other immune cells to leave blood circulation and travel towards the area under the stress. The phagocytic cells, macrophages, dendritic cells and neutrophils, recognize the non-self-antigen by pattern recognition receptors (PRR) residing on the surface of the cell or in the cytoplasm (Farrera & Fadeel, 2015). If the foreign particle is recognized as foreign, it is rapidly engulfed and processed, resulting in release of signalling molecules. Release and type of signals depends from the engulfed matter, and further results in stimulation of other cells of the innate immunity, such as recruitment of monocytes, neutrophils, eosinophils or activation of mast cells. PRR on the surface of the phagocytic cells recognize the intruders' surface molecules, namely pathogen- or damage-associated molecular patterns (PAMPs or DAMPs respectively). PAMPs are molecular structures found from microbes or other pathogenic structures, but not from the host itself. DAMPs in turn, are signal molecules, also referred as "alarmins", resulting from damaged cells or tissue, unusual cell death or stress, and are produced by the host itself. Through the recognition of PAMPs or DAMPs, the cell recruits more specific signals to the surrounding tissue and circulation (Bhattacharya, Andón, El-Sayed, & Fadeel, 2013). In case of pure, inorganic ENM, PAMPs do not exist. Nanomaterial-associated molecular patterns (NAMPs), leading to recognition by innate immune cells, are still not fully identified (Farrera & Fadeel, 2015). Hypotheses of cell recognition

through molecules on the nanomaterial surface (bio-corona) or nanomaterial chemistry itself, have been suggested (Farrera & Fadeel, 2015). ENM might be able to escape the defence mechanisms of the airways. Persistent ENM, cause recruitment of excess amount of immunity cells and mediators to the site, leading in imbalanced immune response in host organism.

### 3.3.1 MACROPHAGES AND DENDRITIC CELLS

Alveolar macrophages have a crucial role in protecting the airways from foreign substances and particles. Residing in lung tissue, they are ready to act when a pathogen or damage-associated molecular patterns are recognized, by engulfing them. They are flexible cells, rapidly internalizing the particle and thus promptly reducing the infection on site (Braciale, Sun, & Kim, 2012).

If macrophages cannot clear and internalize the particles, they release signals and mediators to recruit more innate immune cells. Macrophages secrete several pro-inflammatory cytokines such as tumor necrosis factor (TNF), IL-1, IL-6, IL-8, and IL-12. When the cytokines are produced in appropriate quantities, they are valuable for the host, but become toxic if produced in unregulated manner. IL-10 and TGF-ß in turn, can inhibit macrophage activation and production of pro-inflammatory cytokines, maintaining the balance in inflammatory effects (Arango Duque & Descoteaux, 2014; Martin & Frevert, 2005).

Macrophages are capable to engulf nanomaterials. Whether the particles are completely internalized depends on the size and shape and possibly other properties of the nano-sized particles. It has also been shown, that some nanoparticles, such as Fullerene $C_{60}$ are able to enter the cell with non-phagocytic mechanisms (Fadeel, Pietroiusti, & Shvedova, 2017). If the particle is too big to be engulfed completely, the cell might face frustrated phagocytosis. In this scenario, more macrophages and monocytes are recruited from the blood circulation, causing accumulation of macrophages, and release of excessive amounts of inflammatory signals and alarmins, causing possible immunotoxic effects. Macrophages release also chemokines, leukotrienes, prostaglandins, and complement in response to encountered particles or substances. All of the secreted molecules, can induce increased vascular permeability and recruitment of inflammatory cells, resulting in systemic effects, such as fever (Arango Duque & Descoteaux, 2014).

Dendritic cells are professional phagocytes as well, residing in the tissues and mucosal surfaces, constantly scavenging the surroundings for foreign molecules. They initiate the adaptive immune response by engulfing the particle, processing it, and presenting specific molecules of the pathogen to the T-cells residing in lymph nodes (Braciale *et al.*, 2012). This cascade leading to T-cell activation, makes the dendritic cells a bridge between the innate and adaptive immunity.

### 3.3.2 GRANULOCYTES

Granulocytes are white blood cells of the innate immune system. They fight against the invasion by releasing sets of degrading enzymes from the granules located inside the cell. Granulocyte activation and maturation depends on the inflammatory signals, originating from other immune cells, such as macrophages, epithelial cells or cells going through cell death. The main types of granulocytes are the neutrophils (*circa* 40-70 % of the white blood cells circulating in blood of healthy adults), the eosinophils (1-6 %) and the basophils (1-2 %).

Neutrophils are quick responders and relatively short-lived leukocytes. They are the most abundant leukocytes in blood, rapidly migrating to the site of inflammation after receiving the initiative signal. They either phagocytize the particle and destroy it in intracellular vesicles, or release degradative enzymes stored in their granules (Goncalves, de Liz, & Girard, 2011). These enzymes in turn eliminate or disrupt the invaders, for example, by breaking the protective cell wall of bacteria.

Eosinophils, are recruited from the circulation by specific signals produced *e.g.* by lymphocytes or mast cells. Their main role is to defend the host against parasites, which are usually too large to be phagocytized by macrophages or neutrophils (Murphy *et al.*, 2011). Eosinophils are rarely present in healthy lungs, but are often found from the lungs of asthmatic patients (Simon, Wardlaw, & Rothenberg, 2010).

Several studies have disclosed the recruitment and accumulation of neutrophils and eosinophils to lungs in response to exposure to ENM (Pacurari *et al.*, 2016; Poulsen *et al.*, 2015; 2016; Wang *et al.*, 2013). Since the contribution to the defence is through the release of degrading enzymes, the effects are usually damaging to the host, rather than protective (Murphy *et al.*, 2011).

Mature mast cells are residing in the mucosa, skin and airways, and hence are exposed to inhaled, external particles (Gilfillan & Tkaczyk, 2006). They respond to foreign intruders by degranulation, the release of compounds such as histamine, proteases and several cytokines. This, in turn, cause pulmonary inflammation by recruiting other inflammatory cells. Mast cells play important part in allergic response as well as protecting the host from bacterial and parasite infections (Brown, Wilson, & Metcalfe, 2008; Christy & Brown, 2007). Mast cells seem to be activated by multi-walled carbon nanotube exposure. The hypothesized mechanism involves CNT-triggered cytokine signalling, activating mast cells and thus triggering allergic and asthmatic type responses (Katwa *et al.*, 2012).

## 3.4 HUMAN RESPONSE TO NANOMATERIALS

Several *in vitro* and *in vivo* studies confirm the possible hazardous effects of different types of ENM. However, no methods to predict how *in vivo* and *in*

*vitro* results correlate to human are available to date. Since nanomaterial engineering is a relatively new technology, no large empirical studies of human exposures to indicate their safety risks are yet available. Some case-studies have been published, for example from the Chaoyang Hospital in Beijing, China, where seven print plant female workers were followed up in the period 2007-2008 and reported to experience symptoms such as shortness of breath and pleural effusion. The symptoms were recognized to be caused by inhaled polyacrylate, consisting of nano-sized particles. Pathological examination further confirmed inflammation, fibrosis and foreign body granulomas (Song, Li, & Du, 2009). A health surveillance study conducted in the U.S. by Lee *et al.* in 2015, examined workers manufacturing multi-walled carbon nanotubes. Exhaled breath condensates from the factory workers were found to contain higher levels of oxidative stress markers than the office workers from the same factory (Lee *et al.*, 2015).

Since the recognised hazardous interactions between nanomaterials and the biological systems, the potential adverse effects and their mechanisms need to be thoroughly studied. Currently, the effects of ENM are mainly studied by exposing human or animal cells *in vitro*, or with animal models *in vivo*. It is important that the results from animal exposures are concordant to human exposure scenarios, but there is still lack of standardized testing strategies. For example, *in vitro* and *in vivo* studies are performed with relatively high doses, which cannot be matched to human exposure scenarios. Bio-corona formation, on the other hand, depends from the encountered biological environment and thus affects to the recognition and interactions between cells and ENM, making comparison between animal and human exposures even more difficult. Only small number of ENM exposure studies show characterization of media, whole plasma or blood-particle opsonisation, and how the formed bio-corona affects to the uptake mechanisms by professional phagocytes. Thus, *in vivo* studies are not well supported by *in vitro* models (Gustafson *et al.*, 2015). Despite the difficulty in comparing results from *in vivo* and *in vitro* exposures, certain ENM, such as Graphene Oxide, SiO2, CeO2 and MWCNT, exacerbate similar responses in mouse *in vivo* and *in vitro* (Kim, Boykin, Stevens, Lavrich, & Gilmour, 2014; Snyder-Talkington, Qian, Castranova, & Guo, 2012; Xu *et al.*, 2016). To further compare *in vitro* and *in vivo* responses as well as the differences between human cell lines and murine lungs, also the doses should be adjusted accordingly. At the moment, no common strategies for dose comparison exists, and are currently relying on estimations. In addition, the species-species variation makes the associations between humans and genetically identical animal or cell lines problematic. The immune status and the genetic background of every individual are, in fact, affecting the ENM response. For example in Europe, almost 10 million people (<45 years old) are suffering from asthma (Gibson, Loddenkemper, Sibille, & Lundback, 2013). Vulnerable populations, such as new born, elderly persons or individuals with immunodeficiency or autoimmune disorders should be

considered as separate cohorts when assessing the risks associated with ENM exposure (Farrera & Fadeel, 2015).

## 3.5 BIODISTRIBUTION AND LONG-TERM EFFECTS OF ENM

If nanoparticles are not efficiently cleared from the targeted tissue, a prolonged exposure can lead to chronic inflammation. ENM might cause persistent and detrimental effects to organs and organisms, via similar cascades of events that take place in chronic asbestos exposure. Especially long and fibrous structures are known to remain in different parts of the body for extended period of time, after inhalation or instillation exposures (Jacobsen *et al.*, 2016). High aspect ratio-structures might also remain in the lung pleura, unable to be cleared through stomata, leading to ENM persistence, causing local inflammation and possible mesothelioma, as known in case of asbestos exposure (Donaldson *et al.*, 2013). Long-term exposure studies with rodents, demonstrate high biopersistence of carbon nanotubes, even six months after one-time respiratory administration (Elgrabli *et al.*, 2008; Lohcharoenkal *et al.*, 2013).

Even though ENM might be cleared from the entry site, they might relocate to other districts of the organism. Several studies using rodents indicate biopersistence and distribution of ENM from lungs or gastrointestinal track to blood circulation, and further to organs such as lymph nodes, liver and spleen.

Numerous possible exposure routes, and multiple mechanisms of interactions, as well as different doses and material properties of ENM, makes the study of chronic, and long-term exposure effects unmanageable. As in case of asbestos, detrimental effects of ENM might be detected only several years after the exposure. For this, methods to predict the long-term effects are needed.

## 3.6 NANOMATERIALS AND DISEASES

Nanomaterials have controversial roles: they can trigger inflammatory diseases, but also heal or treat disorders either directly, or by stimulating cells of the immune system when properly controlled (Radomska, Leszczyszyn, & Radomski, 2016). Several nanomaterials have been reported to cause pulmonary inflammation, asthma, chronic obstructive pulmonary disorder and fibrosis in animal models. Main causes for lung injuries after ENM exposure include oxidative stress, inflammation, genotoxicity and fibrosis (Lu, Zhu, Chen, & Liu, 2014). Some studies further suggest that airway exposure to nanomaterials such as $TiO_2$, carbon nanotubes and carbon black might induce systemic cardiovascular toxicity (Lu *et al.*, 2014;

Møller *et al.*, 2016). Because of the inefficient clearance of ENM, the local inflammation in the lungs can progress into systemic oxidative stress and inflammation. Close cross-talk between airways and systemic circulation might also promote cardiovascular dysfunction and heart diseases (Erdely *et al.*, 2009; Lu *et al.*, 2014; Yamawaki & Iwai, 2006).

On the contrary, *e.g.* liposomes, dendrimers, metal particles, peptide-based particles and organic nanomaterials are studied for their possible drug delivery potential (Mirza & Siddiqui, 2014). Some self-assembling nanoparticles are shown to decrease allergic lung inflammation and hyper-responsiveness in asthmatic mice (Kenyon *et al.*, 2013). Another study by Pandey *et al.*, exploiting guinea pigs, indicated positive results in nanoparticle-based drug delivery against *Mycobacterium tuberculosis* (Pandey *et al.*, 2003).

Cytotoxic chemotherapy, is the most widely used anti-cancer treatment. To date, the therapeutic protocols still lack specificity as they indiscriminately target both healthy and cancerous cells, resulting in severe side effects and death of the healthy, fast proliferating cells. Several studies focusing on cancer treatment by ENM drug delivery systems are ongoing. Nanotechnology might provide methods to avoid the damage to healthy cells, by directing the anti-cancer drugs with the aid of ENM, directly to the cancerous cells. Immunotherapy is a rapidly evolving alternative strategy in oncology. It provides a possibility for ENM to initiate and program the cells of immune system against the cancerous cells (Kostarelos, Bianco, & Prato, 2009; Steichen, Caldorera-Moore, & Peppas, 2013). As mentioned above, ENM themselves might trigger granuloma formation, as well as genotoxic effects. For this reason, utilizing ENM in medical applications and in treating diseases is complicated, and a lot of research need to be done for ensuring the safe use of the future nanocarriers and nanodrugs.

# 4 HEALTH RISK ASSESSMENT OF ENGINEERED NANOMATERIALS

Engineered nanomaterials have great potential in several industrial and medical fields, but they might also represent a risk to human health and nature. In order to facilitate innovations in nanotechnology, but at the same time to ensure safe products, better and cheaper methods are needed for prioritization, hazard and final risk assessment purposes. The lack of fundamental knowledge on ENM behaviour in biological systems and their several unique properties, implies that the classical risk assessment strategies do not sufficiently suit to nanoparticles. More knowledge about ENM properties and their biological interactions need to be gathered for enabling a shift towards more predictive risk assessment (RA) strategies.

## 4.1 TRADITIONAL RISK ASSESSMENT

The current risk assessment strategy for a chemical substance is based on regulatory approved laboratory tests (*in vivo* and *in vitro*) by which the target organ toxicity and the no-observed-adverse-effect-level (NOAEL) are clarified. Further information needed is chemical substance´s absorption, distribution, metabolism and excretion combined with toxicokinetics and pharmacokinetics information. The main outcome of RA is to identify the sources of uncertainties, state the degree of harm to target organisms or systems, and to define the limits for safe use. This toxicity testing strategy for chemical substances is mainly animal testing based. Conventional RA is time consuming and exhausting procedure, requiring substantial amount of background information and exposure testing.

Due to the complex nature of nanomaterials (size, charge, functionality, transformation *etc.*) and large amount of test material needed, animal tests are not in general feasible for ENM toxicity testing. Therefore, novel, predictive computational tools, systems biology methods and human cell based *in vitro* approaches may complement traditional risk assessment. This strategy also diminishes or replaces extensive animal testing.

To protect human health and the environment, the European Union is controlling the use and production of all the chemical substances imported or produced in the EU, by regulation called REACH (Registration, Evaluation, Authorization and Restriction of Chemicals). The law requires manufacturers and producers to register and systematically control the production and distribution of all the chemicals. Substances need to go through systematic risk assessment procedure, including i) hazard identification, ii) hazard assessment, iii) exposure assessment and iv) risk assessment (The European

Chemicals Agency, 2009). **Figure 4** presents an example of the risk assessment process to industrial chemicals under REACH.

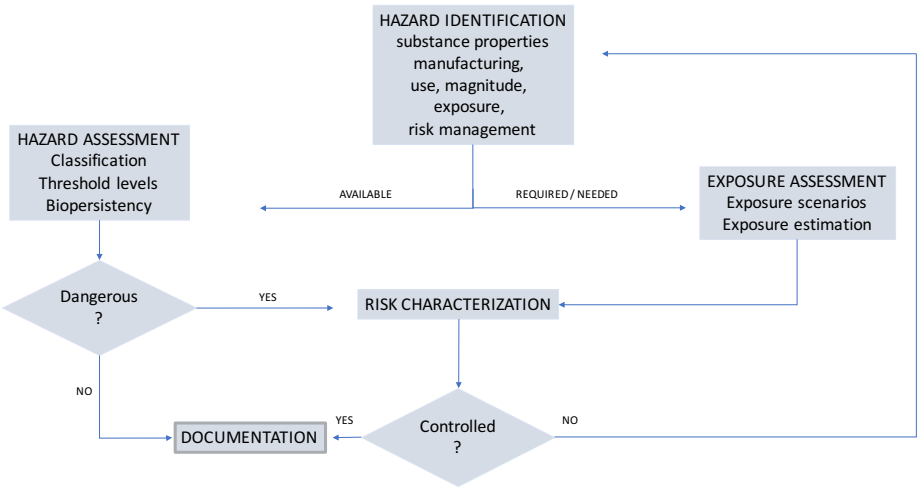


**Figure 4** Traditional risk assessment process under REACH.

All substances, regardless of their use and applications, need to be safe to humans and with manageable risk to the environment. Currently, the situation is complicated by the fact that the risk assessment process varies between different regulations and directives in force for specific classes of compounds, such as cosmetics, biocidal products, plastic materials, industrial emission, medical devices and food and feed additives (Heinonen, Louekari, & Tähti, 2014).

As nanomaterials belong to all of the aforementioned groups, it is evident that the hazard assessment of all ENM independently will be unmanageable with the current approaches. First, not enough data and knowledge for defining possible hazardous potential of the ENM is available. Second, all the possible ENM properties and property-combinations that could affect the toxicity are still poorly understood. Third, ENM affect humans and the environment at global scale, instead of smaller sub-populations, thus all the possible exposure scenarios are not yet recognized. Fourth, we lack essential understanding on how ENM interact with living systems, and how their behaviour changes during time and in different biological environment. In addition, time consuming risk assessment cannot keep up with the pace that new ENM are being developed. REACH has also been criticized for the excessive number of required animal testing. To date, marketing of novel cosmetic products which hazard assessment is based on animal testing, is forbidden in Europe (Henkler *et al.*, 2012). Thus, proposals to move towards *in vitro*, *ex vivo and in silico* exposures, combined with high-throughput and omics methodologies, and predictive computational models have been

suggested for nanomaterial safety and toxicity testing (Hartung & Rovida, 2009; Nel *et al.*, 2013).

## 4.2 TOXICOLOGY IN THE 21ST CENTURY (TOX21)

Several new strategies are proposed for the shift towards predictive risk assessment and to cut down the number of animal experiments used in classical toxicology testing. For example, TOX21, a federal collaboration for moving towards predictive discipline was formalized in 2008, in collaboration with the U.S National Institute of Environmental Health Sciences and the U.S. National Center for Advancing Translational Sciences, U.S. Environmental Protection Agency, and U.S. Food and Drug Administration. The overall goal of TOX21 is:

"*To research, develop, validate, and translate innovative test methods that will better predict how chemicals may affect humans and the environment. Results from these methods will be used to:*

1. *Prioritize substances for further in-depth toxicological evaluation*
2. *Identify mechanisms of action for further investigation (e.g., disease-associated pathways)*
3. *Develop models that better predict how chemicals will affect biological responses (predictive toxicology)"* (National Research Council, 2007; Tice, Austin, Kavlock, & Bucher, 2013)

By utilizing the most modern tools available in the fields of biology and chemistry, together with omics and high-throughput screening methods, TOX21 aims at converting classical toxicology and risk assessment into more rapid and cost-effective regulation approaches. In addition, TOX21 aims at providing animal-free approaches (3R) for toxicity testing (National Research Council, 2007).

## 4.3 REDUCTION, REFINEMENT AND REPLACEMENT OF ANIMAL TESTING

Classical toxicology approaches rely on extensive animal testing, aiming to identify the hazard potential of a chemical to humans, to target organ and finally to assess the risk. In the regulatory testing both rodents and non-rodents are needed. Of rodents, rat is the preferred animal although mouse can also be used. Of non-rodents, dog or mini pig are the species mostly used. The environmental safety is assessed using organisms such as crustaceans, earthworms and zebrafishes. Even though animal studies are still the paramount method to understand toxicity and health risks of a chemical, they are also expensive, technically demanding, time-consuming

and ethically questionable. Several disadvantages and suffering of the animals led to the development of 3R (Refinement, Reduction and Replacement of animal testing) principle in year 1959 (Russell & Burch, 1959). The main aim of the 3R, is to encourage the scientific community to develop substituting techniques, as well as to increase the quality of *in vivo* tests and improve animal welfare in tests (Burden *et al.*, 2017).

Even though animal tests cannot be completely replaced, at least in the near future, complementing *in vitro* testing methods are proposed and designed. The current problem with *in vitro* toxicity testing especially with nanoparticles, is the lack of global standards and testing strategies that would guarantee comprehensive, comparable and valid outcomes, as well as reliable concordance between *in vitro* and *in vivo* strategies. Especially in long-term, chronic exposure studies, feasible *in vitro* methods are not available. On the other hand, the predictivity of an animal model to human is also questioned (Heinonen, 2015). As nanotechnology is a relatively new field, reliable and validated testing strategies as well as meaningful doses and time-points are also not yet available. Thus, the gained results from animal exposures coming from different laboratories and research institutes might not be in concordance with each other. The ethically debated animal testing is still the only inclusive way to test hazardous and toxic potential of chemicals. Common, universal strategy for ENM toxicity testing would reduce the number of animals and repetitive testing. *In vitro* exposure methods, utilizing 3D cell cultures, co-cultures or tissue engineering cultures, would provide alternative approaches for exposure assessment. Predictive computational methods and mathematical models could be further utilized in minimizing the need of animal testing. Altogether, enormous amount of work and common testing strategies is still required to achieve reliable and comparable methods for replacing animals as a model organism.

## 4.4 SYSTEMS TOXICOLOGY APPROACHES FOR ENM SAFETY ASSESSMENT

Systems toxicology is an integrated approach to assess hazard and risk of a substance. It combines methods and knowledge from chemistry, cheminformatics, biology, bioinformatics, statistics, and mathematics, to examine and model complicated and dynamic biological systems (Sturla *et al.*, 2014). Systems toxicology aims at developing and utilizing up-to-date computational strategies for outlining the toxic potential of a substance. Mathematical models are developed and used to measure and predict the outcomes and effects of exposures. Comprehensive mapping of all changes occurring in biological environment, including responses of genes, proteins, metabolites, signals *etc.* in the context of an exposure, would allow building more thorough picture of the substance mode of action (MoA). For this to happen, inclusive, comparable and high-quality data from *in vivo* and *in*

*vitro* exposures need to be produced, as well as appropriate computational tools, that would be specifically designed to take into account the complexity of a substance.

The current risk assessment approach has several limiting factors and weaknesses, leading to severe knowledge gaps: appropriate exposure doses, human-relevant exposures set-ups, biophysiological differences between organisms, biological thresholds, end-points and population variability (Sturla *et al.*, 2014). To overcome the existing concerns, systematic, computational approaches are being developed and exploited. Systems toxicology has the potential to provide mechanistic tools and models to overcome the before mentioned limitations. In case of ENM, we are still in need of essential understanding of how nanomaterials interact with the environment. Applying novel systems biology tools to already existing ENM-exposure data might facilitate the identification of ENM-specific responses and characteristics, and the discovery of novel biomarkers. Predictive, computational approaches would aid in establishing *in vitro* and *ex vivo* tests of toxicity and final risk assessment instead of using *in vivo* tests (Vinken, 2013).

Because of the shortfalls and complexity of ENM, we are currently unable to recognise all the relevant doses, interactions and end-points of ENM that might lead to toxic potential. Several large EU-funded projects, such as NANOSOLUTIONS (http://nanosolutionsfp7.com), NANOMILE (http://nanomile.eu-vri.eu), MARINA (http://www.marina-fp7.eu), NanoValid (http://www.nanovalid.eu), NANOMMUNE (http://www.nanommune.eu), caLIBRAte (http://www.nanocalibrate.eu), and SmartNanoTox (http://www.smartnanotox.eu) are aiming to in-depth understanding of nanomaterial behaviour, interactions and responses of biological entities in well-controlled manner. For achieving greater level of knowledge, the projects are integrating classical exposure settings with systems biology approaches (Savolainen *et al.*, 2013).

Different computational models and intellectual frameworks for risk assessment are being developed: (Quantitative) Structure-Activity Relationship (QSAR), is a statistical model aiming at identifying key features of the substance, that could be used to predict its toxicity (Burello & Worth, 2011). This approach is based on the assumption that structural descriptors, such as shape, charge *etc.*, correlate with a particular biological activity of interest, and thus can be used to predict the interactions between the organisms and the substance (Jagiello *et al.*, 2016).

Directly derived from the QSAR reasoning is the read-across strategy, where the known endpoint information of one chemical is used to predict the endpoint of another structurally similar chemical. It is utilized to fill data gaps of the effects within same group of substances, in other words, substances presumed to have highly similar physicochemical, toxicological and ecotoxicological properties, enabling the grouping of substances under category labels (Torges, 2013). Several grouping and classification strategies

are proposed also for the ENM: i) dimensionality, shape and morphology; ii) composition and chemistry; iii) complexity and functionality; and iv) biointerface.

Because of the variety of ENM features and lack of measuring techniques, as well as overlap between several categories, no single classification method has been identified (Savolainen *et al.*, 2013). To recognise the hazardous effects of nanomaterials, the above mentioned computational strategies for RA, are also being considered and suggested to overcome the limitations of studying the descriptive impact of every ENM individually.

Instead, the adverse outcome pathway (AOP) approach is aiming at recognising all the sequential events leading to adverse health or ecotoxicological consequences (**Figure 5**). It considers the molecular, cellular, organ, organism and population responses as well as the properties of the chemical. AOP aims at providing clear mechanistic representation of critical toxicological effects and is considered to have a great potential in predictive toxicology (OECD, 2016; Vinken, 2013).

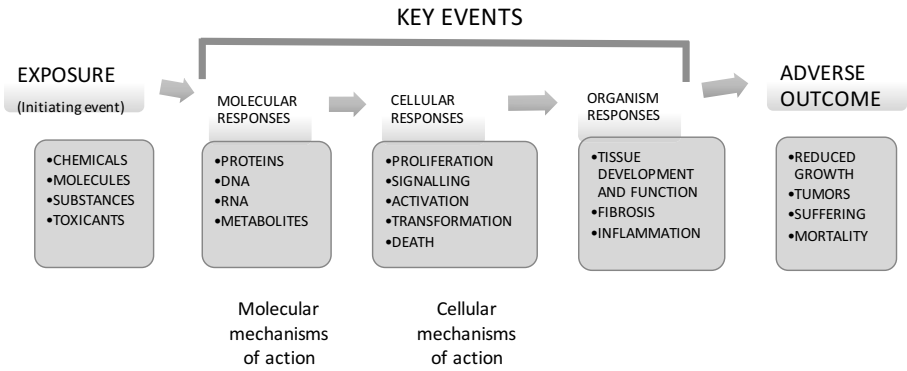


**Figure 5** Scheme of the Adverse Outcome Pathway structure

In addition to chemical substances, the definition of AOP can also provide valuable information to the nanomaterial risk assessment. However, several layers of information need to be collected and integrated in order to reconstruct reliable AOP. To date, enormous amount of ENM exposure data

are being produced, but the methodologies for processing the data are in continuous development, as more advanced computational strategies are needed for the interpretation of the data, thus moving towards predictive hazard assessment.

## 4.5 OMICS APPROACHES IN NANOTOXICOLOGY

ENM are likely to cause changes in the molecular patterns of the exposed organism. Large scale assays, such as DNA microarrays, mass spectrometry and high throughput sequencing methods are utilized to inspect the molecular alterations happening during exposure, offering a perspective on the mechanistic changes occurring in cells or tissues. Since several biological factors are affecting the exposure outcome, multiple aspects must be taken into account when building comprehensive understanding of the ENM mechanism of action (MOA): genetic (genomics), epigenetic (epigenomics), transcriptional (transcriptomics), proteic (proteomics), and metabolic (metabolomics) variations (Marx-Stoelting *et al.*, 2015).

Omics methodologies are currently widely used in quantifying and producing information about molecular changes of an organism (Jennen *et al.*, 2011; Robinson, Pennings, & Piersma, 2012; Tralau *et al.*, 2015). Systems toxicology aims at pinpointing the functional and molecular level changes in respect to exposures and, in addition, to gain wider standpoint of all the events that lead to specific responses. Thus, omics methods provide great tool in achieving deeper understanding of ENM effects, which could be expanded to cover also the risk assessment of ENM. In order to construct systematic toxicological phenotypes and MoA in respect to ENM exposure, computational approaches to strategically combine the different data layers (molecular, functional and anatomical changes) need to be established.

### 4.5.1 TRANSCRIPTOMICS

The term transcriptome is referred to as virtually all the RNA molecules transcribed from a given genome, and transcriptomic technologies allow the profiling of many RNA transcripts in the same assay. Expression profiling is one of the most recognised method to identify biological changes and specific responses associated to exposure or disease (Dopazo, 2014; Sturla *et al.*, 2014).

DNA microarrays are a widely-established technology based on nucleic acid hybridization to compare gene expression levels across several biological conditions. Similarly, next generation sequencing (NGS) is using the sequencing of nucleic acid molecules to produce a digital representation of the transcriptional activities (Sturla *et al.*, 2014). Both methods offer valuable information of the RNA-level changes and provide large quantities

of data stored in public databases to be utilized by the scientific communities. Studies comparing the two methods are showing good concordance in terms of expression profiling in different disease and exposure conditions (Guo *et al.*, 2013; C. Wang *et al.*, 2014).

When the transcriptome data are analysed properly, it can provide insight into transcriptional mechanism of action (TMOA) in response to a certain stimulus. Computational methods are also available to further exploit transcriptomics data in search of biologically meaningful responses, enriched pathways, interconnections and biomarkers. Several available databases are covering a wide variety of gene annotations and profiling information. Available databases provide mostly computationally curated, static information about genes and their interactions. Nevertheless, they are valuable tools, enabling mining of huge transcriptome datasets, to find more specific responses, and more in-depth interpretation of the biological and molecular processes.

## 4.6 PATHWAYS OF TOXICITY

In year 2007, the U.S. National Research Council (NRC) proposed a strategic plan for the toxicity testing in the 21st century, envisioning new approaches for predictive and high-throughput *in vitro* assays to evaluate pathways of toxicity (PoT) (National Research Council, 2007). PoT are patterns of molecular alterations representing relevant cellular processes mediating adverse outcomes of toxicants (Kleensang *et al.*, 2014). A pathway-based approach is hypothesized to provide mechanistic understanding of the initiating events and adverse outcomes, as well as identifying novel biomarkers (Kleensang *et al.*, 2014).

Currently, databases dedicated for toxicological pathways *per se* do not exist, but suggestions for building PoT database have been proposed (Kleensang *et al.*, 2014). For example, The Human Toxome Project, started in 2016, aims at developing tools for pathway mapping annotation and validation by systems toxicology (Bouhifd *et al.*, 2014). To fully recognise the toxicity pathways, data should be inclusive, incorporating phenotypic and mechanistic effects, exploiting complementary omics technologies, such as proteomics and transcriptomics. To get comprehensive understanding of the toxicants effects, more unified and standardized toxicity testing approaches should be developed. Creation of PoT database would require large coordination commission, as well as commitment and collaboration between toxicologists, regulators and scientific community. It also involves development of structured vocabulary and ontology engineering, as well as development of new systems biology tools and concepts. PoT would benefit systems toxicology based risk assessment approaches also in respect to nanotoxicology, by providing information about the ENM exposures

outcomes also *in vitro*, as well as *e.g.* in building more comprehensive biological networks about the specific responses.

## 4.7 BIOLOGICAL NETWORKS

Exposure and disease conditions likely cause changes in multiple molecular districts of an organism. With up-to-date omics technologies, these changes can be measured and documented (Costa & Fadeel, 2016; Hood, Heath, Phelps, & Lin, 2004; Kamburov, Pentchev, Galicka, Wierling, Lehrach, & Herwig, 2011b). However, understanding the relevance and biological meaning underlain by the large data sets represents an important challenge. In this sense, the approach based on graph theory, or network approach, provides a powerful way to examine and interpret complicated mechanistic effects. Biological networks (BN) are model representations of complex interactions, in which nodes, *e.g.* genes, proteins or other molecules, are interconnected to each other by edges, which are a function of the degree of relatedness. Networks can be constructed from different entities and data layers, integrating experimental data with prior knowledge. Transcriptomics data, for instance, are a viable substrate to infer gene networks where the edges represent the degree of co-expression of genes across different experimental conditions, under the assumption that co-expressed genes are likely to be co-regulated and to cooperate to the same biological functions (Serin, Nijveen, Hilhorst, & Ligterink, 2016). Once inferred, large networks can then be mined to find specific communities of molecules highly interconnected to each other as well as significantly altered in a condition of interest. BN models could provide a computational strategy to ENM toxicology, to recognise and predict specific, but complex molecular interactions and alterations after ENM exposure both *in vivo* and *in vitro* (Sturla *et al.*, 2014).

## 4.8 NANOTOXICOLOGY: STANDPOINT FOR FUTURE

Up-to-date omics approaches can generate humongous amount of data, but we still need to discover the biological meaning behind the data (Hu, Li, Gao, Mu, & Zhou, 2016). Several publicly available databases are enabling to mine information about the biological processes and functions behind the large data sets. Nevertheless, all databases have their downsides and limited number of representations. Pathways are not build on toxicology *per se*, and thus comprehensive interpretation and causality are still left unknown. New computational methods to supplement (or replace) traditional toxicology approaches are being developed and tested to get deeper understanding of the data. Novel, validated approaches are also essential for storing and collecting the generated toxicity data.

Currently, data from ENM exposures and studies is scattered in different databases, usually project by project. Combining all the available information about ENM exposures, would be essential for creating comprehensive source for ENM effects and toxicity outcomes (Savolainen *et al.*, 2013). Unfortunately, common testing strategies do not exist. Harmonization of the exposure data would facilitate the risks assessment procedure, data mining, as well as helping researchers to develop predictive tools.

There is no simple way to generate solutions to the existing challenges in nanotoxicology, and integration of new knowledge to already existing databases in respect to ENM exposure is not easy.

Developing new systems toxicology based tools for nanomaterial toxicity testing, would not only benefit the nano-community, but also toxicologists, and consequently also industrial sectors. Computational approaches together with *in vitro* and *ex vivo* testing, would further diminish extensive animal experiments and suffering. However, many gaps need to be still filled before new computational toxicology assessment could be beneficial in regular basis (Nel, 2013).

When the properties of ENM that cause the hazard are well recognized, we would be able to produce new generation of ENM, materials that would be safe by their design. Developing safe materials, would have world-wide impact to industry, benefitting both human and nature, as well as reducing animal exposures in toxicity testing (Halappanavar, Vogel, Wallin, & Yauk, 2017).

# 5 AIMS OF THE STUDY

Knowledge about the hazard potential of engineered nanomaterials is urgently needed. Classical hazard assessment is time consuming, often requiring extensive animal tests. Continuous expansion of nanotechnology as well as the several unique characteristics of ENM are complicating the risk assessment and predictions of the nanomaterials behaviour and safety. Appropriate, standardized methods for testing ENM in effective manner are still missing. New approaches to predict human health hazard are needed for adequate risk assessment.

The overall goal of this thesis is to utilize systems toxicology approaches to support and simplify the ENM hazard assessment.

The specific aims of this thesis are:

1. To evaluate the concordance between two different *in vivo* ENM pulmonary exposure methods in mice.
2. To assess systems biology-based strategies to the study of ENM mechanisms of action (MOA).
3. To characterize the CNM MOA associated to their intrinsic properties in *in vivo* and *in vitro* exposure set ups.

# 6 MATERIALS AND METHODS

**Table 1** Summary of the materials and methods, and the corresponding publications

| Materials / Methods | Publication |
|---|---|
| Carbon Nanomaterials | Ⅰ, Ⅱ |
| Particle characterization | Ⅰ |
| Animals | Ⅰ, Ⅱ |
| *in vivo* exosure (Whole body inhalation method) | Ⅰ |
| *in vivo* exposure (Oropharyngeal asiration) | Ⅰ, Ⅱ |
| *in vitro* exposure (THP-1 Cell line | Ⅱ |
| Sample collection and preparation (*in vivo*) | Ⅰ, Ⅱ |
| Sample collection and preparation (*in vitro*) | Ⅱ |
| Histology | Ⅰ, Ⅱ |
| Bronchoalveolar lavage | Ⅰ, Ⅱ |
| Light microscopy | Ⅰ, Ⅱ |
| RNA extraction and purification | Ⅰ, Ⅱ |
| complementary DNA synthesis | Ⅰ |
| reverse transcription quantitative PCR | Ⅰ |
| Transcriptome assays (DNA microarrays) | Ⅰ, Ⅱ |
| DNA microarray computational analysis | Ⅰ, Ⅱ |
| Statistical methods | Ⅰ, Ⅱ |
| Enrichment analysis | Ⅰ, Ⅱ |
| Network Inference | Ⅱ |

## 6.1 CARBON NANOMATERIALS AND PREPARATION FOR EXPOSURES (I, II)

The focus of this thesis, is on *in vitro* and *in vivo* exposures to different sized and shaped carbon nanomaterials. The complete list of the used methods and carbon nanomaterials (CNM) with corresponding properties and vendors are listed in **Table 1** and **Table 2** respectively.

Altogether six CNM, including tubular, spherical and fibrous structures were exposed to mice and PMA-differentiated THP-1 macrophage-like cells. For both exposure set-ups, aspiration and *in vitro*, the CNM-suspensions were freshly prepared, by weighing the CNM and adding corresponding amount of PBS (for *in vivo*) or media (for *in vitro*) to obtain 1mg/mL stock solution, following 20-minute sonication and rigorous vortexing. Further dilutions for exposures were prepared from the stock, vortexed and mixed to

avoid agglomeration and sedimentation of the CNM. Inhalation exposures were performed by aerosolizing the dry rCNT powder in the exposure chamber.

**Table 2** Carbon nanomaterials studied and their properties.

| Material Acronym | rCNT | tCNT | Baytube | Graphite | Fullerene | SES |
|---|---|---|---|---|---|---|
| Description | long rigid multi-walled carbon nanotube | long tangled multi-walled carbon nanotube | short tangled multi walled carbon nanotube | long rigid carbon fibre | hollow carbon sphere | short rigid multi-walled carbon nanotube |
| Product code | MWCNT-7 mitsui | Cheaptubes | Baytubes C150 HP | 636398 | MTS60 | 900-1260 |
| Provider | Mitsui & Co. | Cheaptubes Inc. | Bayer Material Sci. | Sigma-Aldrich | MTR Ltd. | SES research |
| Shape | tube | tube | tube | fiber | sphere | tube |
| Aspect Ratio | 2.6 | 26.09 | 0.69 | 0.71 | 0.01 | 0.75 |
| Avg. length (nm) | 13 000 | 30 000 | 1 000 | 10 000 | 100 | 2 000 |
| Avg. Diameter (nm) | 50 | 11.5 | 14.5 | 140 | 100 | 20 |
| Avg. Surface Area (m2/g | 22 | 180 | 204 | 32 | 20 | 60 |
| Publication | I , II | II | II | II | II | II |

## 6.2 PARTICLE CHARACTERIZATION

### 6.2.1 ELECTRON MICROSCOPY (I, II)

Particles have been extensively characterized earlier by Vippola *et al.* 2009 (Vippola *et al.*, 2009).

In addition, morphology of the rCNT was investigated by field-emission electron microscopy (SE, Zeiss Sigma HD-VP) and transmission electron microscopy (TEM, Jeol JEM-2100F).

### 6.2.2 ZETA POTENTIAL (I)

To examine the stability of the rCNT dispersions, electrokinetic potential was inspected with electrophoretic mobility analysis (ZetaSizer ZS DLS, Malvern Instruments) from freshly prepared rCNT-suspensions.

### 6.2.3 RAMAN SPECTROSCOPY (I)

To analyse the composition of the rCNT suspensions, a small drop of the dilution was pipetted onto a glass slide, and let to dry before examination by Raman spectroscopy (Bruker Senterra 200 LX).

## 6.3 ANIMALS

Female C57BL/6 mice (7 to 8 weeks old, 6-9 mice per exposure group) were exposed to all the six CNM (Scanbur A/S, Karslunde, Denmark) and to PBS

as controls. Animals were randomized to groups of four, housed in stainless steel cages with 12-hour dark/light cycles. Mice were quarantined for 1 week after arrival, and provided with standard mouse chow diet (Altromin no. 1314 FORTI, Altromin Spezialfutter GmbH & Co., Germany) and water *ad libitum*. The room temperature was kept between 20 and 21 °C, and humidity between 40 and 45 %. All experiments were performed in agreement with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg March 18, 1986, adopted in Finland May 31, 1990). The study was approved by the Animal Experiment Board and the State Provincial Office of Southern Finland.

## 6.4 EXPOSURE *IN VIVO* (I, II)

### 6.4.1 WHOLE-BODY INHALATION (I)

Inhalation exposure to rCNT was performed by Rydman and Ilves *et al.* 2014 (Rydman *et al.*, 2014). rCNT aerosol was achieved by fluidized bed aerosol generator (FBAG; TSI Model 3400A). Mice were freely inhaling the aerosolized dry rCNT powder in a whole-body inhalation chamber, four hours per day, for four consecutive days. Aerosol concentrations ranged from 6.2 to 8.2 mg/m$^3$. Untreated, control mice, were housed in same room with the rCNT-exposed mice, but were not moved to exposure chamber, due to possible rCNT contamination risk. Mice were sacrificed 24 hours after the final exposure.

### 6.4.2 OROPHARYNGEAL ASPIRATION (II)

Freshly prepared CNM suspensions (200 and 800 μg/mL of PBS), either supplemented with 0.6mg/mL of BSA (I), or plain PBS (II), were prepared from 1mg/mL stock solutions and sonicated for 20 minutes just prior to use. Mouse was anesthetized with vaporized isoflurane (Isoflurane Baxter, U.S.) and moved onto custom made aspiration stand. Tongue was gently pulled out with tweezers, and 50 μL of the freshly prepared CNM suspension was pipetted onto the back of the tongue. Nose was closed, forcing the mouse to aspirate the CNM. Procedure was repeated on four consecutive days, and mice were sacrificed 24 hours after the final exposure. Control mice were aspirating 50 μL of BSA/PBS (I) or plain PBS (II) (**Figure 6**).

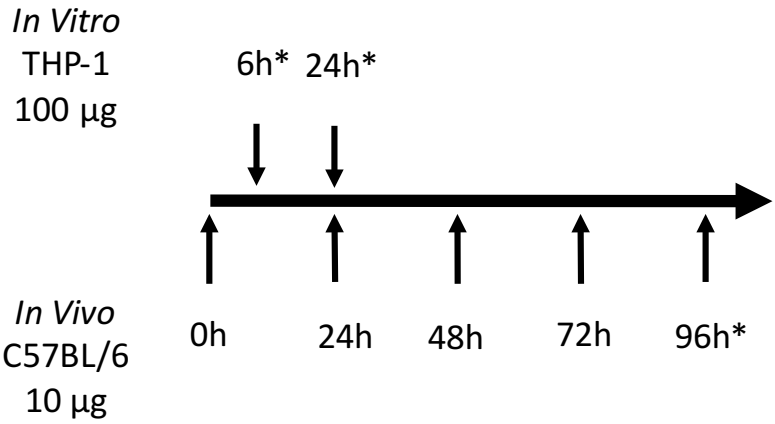


**Figure 6** Experimental design of the exposures *in vitro* (II) and *in vivo* (I and II). Asterix (*) denote the time points of sample collection.

## 6.5 EXPOSURE *IN VITRO* (II)

THP-1 cells (ATCC TIB-202) were grown in cell culture flasks, in RPMI 1640 media (Gibco, Thermo Fisher Scientific) supplemented with 10% FBS, 1% penicillin-streptomycin antibiotics and 2 mM ultraglutamine. Cells were divided to six-well plates (1.5 x $10^6$cells/well) and differentiated with 50 nM PMA (phorbol-12-myristate-13-acetate) for 48 hours. Freshly made CNM stock solutions of 1 mg/mL were prepared to complete RPMI media, vortexed and sonicated for 20 minutes in bath sonicator (Elmasonic S15H, Ilabequipment, New Jersey, USA). Further dilutions of 100 µg/mL were made to complete RPMI media, vortexed vigorously and sonicated in bath sonicator for additional 20 minutes. 1mL of CNM suspension was pipetted on top of the cells, incubated for 6 or 24 hours in +37°C. Untreated, PMA-differentiated cells were used as controls (**Figure 6**).

## 6.6 SAMPLE COLLECTION AND PREPARATION (I, II)

### 6.6.1 *IN VIVO* (I, II)

Twenty-four hours after the final exposure, mice were sacrificed, lungs were lavaged with 800 µL of PBS by cannulating the trachea with syringe for 15 seconds. Cells from bronchoalveolar lavage (BAL) were fixed onto microscope slides and stained with May Grünwald-Giemsa staining solutions. Right lung was fixed with formalin, embedded in paraffin, cut, affixed on slides and stained with H&E and acid-Shiff (PAS) staining

solutions. The left lobe was cut and divided into sub-samples into RNAlater solution (Ambion, Life Technologies, CA, USA), and stored at -70 °C.

### 6.6.2 *IN VITRO* (II)

After exposures for 6 or 24 hours, the exposed cells were harvested and lysed, and total RNA was extracted and purified with Qiagen RNeasy plus mini kit according to instructions provided by vendor (Qiagen, GmbH, Hilden, Germany). RNA samples were stored at -70 °C.

## 6.7 HISTOLOGY (I, II)

H&E and Picrosirius Red –stained tissue samples were visually examined and evaluated. Mucin producing Goblet cells, were counted from Periodic Acid Shiff –stained lung tissue sections, by calculating the activated cells from surface area of 200 μm.

## 6.8 BRONCHOALVEOLAR LAVAGE (I, II)

BAL liquid was cytocentrifuged onto glass slides and stained with May Grünwald-Giemsa (MGG) stain. Macrophages, neutrophils, eosinophils and lymphocytes were counted from three high-power fields (HPF) under light microscope (Leica DM 4000B, Leica, Wetzlar, Germany) and the counts were averaged.

## 6.9 LIGHT MICROSCOPY (I, II)

Light microscopy was utilized in counting macrophages, neutrophils, eosinophils and lymphocytes from MGG-stained BAL samples. Activated Goblet cells were counted from PAS-stained lung sections, and histological evaluation was performed from H&E and Picrosirius Red-stained lung tissue samples. Magnifications were varying from 100 to 500x.

## 6.10 RNA EXTRACTION AND PURIFICATION (I, II)

### 6.10.1 *IN VIVO*

Lung samples stored in RNAlater in -70 °C, were thawed and moved to lysing matrix D tubes (MP Biomedicals, Illkirch, France) containing TRIsure reagent (Bioline reagents, Ltd., London, UK). Samples were homogenized in

FastPrep FP120 homogenizer (BIO 101, Thermo savant, Waltham, MA, USA). RNA was extracted and purified based on phenol-chloroform extraction protocol by Bioline Reagents. Nanodrop (ND-1000, Thermo Fisher Scientific Inc., Wilmington, NC, USA) was used for quantifying the yield and confirming the RNA purity. Bioanalyzer (Agilent Technologies, USA) was further utilized for RNA quality control. Samples with RNA integrity value (RIN) >8 were used in DNA microarray analysis.

### 6.10.2 *IN VITRO*

Total RNA was extracted and purified with Qiagen RNeasy plus mini kit according to instructions provided by vendor (Qiagen, GmbH, Hilden, Germany). Purified RNA samples, stored in -70 °C, were thawed and the RNA quality was confirmed by Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Samples with RNA integrity number (RIN) >9 were used for DNA microarray analysis.

## 6.11 COMPLEMENTARY DNA SYNTHESIS (I)

Complementary DNA (cDNA) was synthesized from 500 ng of total RNA in a 25 µL reaction, with MultiScribe reverse transcriptase and random primers (The High Capacity cDNA Archive Kit, Applied Biosystems). Synthesis was performed according to the manufacturer's instructions, in a 2720 Thermal Cycler (Applied Biosystems, Carlsbad, CA, USA) with thermal cycles of 25°C for 10 min and 37 °C for 120 min (Thermal Cycler, Applied Biosystems).

## 6.12 REVERSE TRANSCRIPTION (REAL-TIME) QUANTITATIVE PCR (I)

RT-qPCR was performed to quantify the expression changes of cytokine IL-13. Corresponding probes and primers (18S rRNA and IL-13, Applied Biosystems, Foster City, CA, USA) were utilized for relative quantification 7500 Fast System (7500 Fast Real-Time PCR system, Applied Biosystems). Amplification was performed in 11 µL reaction volume, containing 1 µL of cDNA sample, TaqMan universal PCR master mix and primers. Results were normalized against 18S ribosomal RNA housekeeping gene, and relative expression levels were calculated between treated and control groups.

## 6.13 TRANSCRIPTOME ASSAYS (DNA MICROARRAYS) FROM *IN VIVO* SAMPLES (I, II)

Two lung RNA samples from same experimental group, with RIN-value >8 were pooled together. AffinityScript RNase Block (Quick Amp Labeling Kit, Two-color, Agilent) was used for converting oligo-dT (T7) primed sample RNA as complementary DNA. T7 RNA polymerase was used for amplification of fluorescently labelled (Cy3 or Cy5) cDNA samples. Labelled samples were cleaned by Qiagen RNeasy mini spin columns (Qiagen, GmbH, Hilden, Germany) and fragmented cRNA was hybridized to Sure Print G3 Mouse GE 8 x 60K arrays (Agilent Technologies, Two-Color Microarray-Based Gene Expression Analysis, Low Input Quick Amp Labelling). Microarray slides were scanned with Agilent microarray scanner (Agilent Technologies, DNA Microarray Scanner with Surescan High-Resolution Technology, model G2505C, USA) and data was extracted using Agilent Feature Extraction Software (V11.0.1.1). Linearity, sensitivity and accuracy was monitored by hybridizing predefined transcripts to control probes (Agilent Spike-In Kit). Spot intensities were calculated as the median foreground intensities.

## 6.14 TRANSCRIPTOME ASSAYS (DNA MICROARRAYS) FROM *IN VITRO* SAMPLES (II)

Antisense RNA (aRNA) copies were synthesized using T7 RNA polymerase (amino allyl MessageAmp II aRNA amplification kit, Ambion, Carlsbad, CA, USA). aRNA samples were labeled with monoreactive Cy3, Cy5 (GE Healthcare, Buckinghamshire, UK), or Alexa 488 (Invitrogen, Eugene, Oregon, USA). Samples were purified and hybridized onto Agilent human GE 4×44K V2 microarray slides (Agilent Technologies, USA). Slides were washed (Agilent gene expression wash buffers 1 and 2) and scanned with GenePix 4200 AL (Molecular Devices, Sunnyvale, CA, USA). Image segmentation was done by GenePixPro 6.1 software (Molecular Devices). Spot intensities were calculated as the median intensities.

## 6.15 DNA MICROARRAY COMPUTATIONAL ANALYSIS (I, II)

Quality of the mouse arrays was confirmed from the quality reports provided by Agilent Feature Extraction Software (V11.0.1). Data was imported to R software (R Development Core Team, 2011) and the median intensities were log2 transformed and quantile normalized with Bioconductor package Limma (Ritchie *et al.*, 2015). SVA -package with Combat function was utilized for removal of batch effects, emerging from labelling and slide variance (Leek, Johnson, Parker, Jaffe, & Storey, 2012). Linear model was fitted to the batch-corrected data and pairwise comparisons were performed

by empirical Bayes method. Genes with linear fold change >|1.5|, and Benjamini-Hochberg adjusted P-value < 0.01, were considered significantly differentially expressed. Microarray datasets have been deposited in NCBI Expression Omnibus (GEO) database and are accessible through GEO series accession numbers GSE85711 (I) and GSE92901 (II).

## 6.16 STATISTICAL METHODS (I, II)

A nonparametric Mann-Whitney U test was used to evaluate the statistical significance between the cell counts of the exposed groups, presented as mean and standard error of the mean. P-value <0.05 was considered statistically significant. (I and II)

Correlation scores between each gene and CNM property were computed by averaging correlation scores from Pearson, Spearmann and Kendall methods. The average correlation value was multiplied with *–log(P-value)* for attaining a robust correlation score to each gene-CNM property pair. (II)

## 6.17 ENRICHMENT ANALYSES (I, II)

A set of enrichment analysis tools were used to examine activated pathways, Gene Ontology-terms and biological processes in response to CNM exposures. Differentially expressed genes were used as an input to retrieve the enriched pathways and biological processes.

R-package BACA was used to query the pathways from Kyoto Encyclopedia of Genes and Genomes (KEGG) through DAVID database (Huang *et al.*, 2007), and further to build bubble plots to visualize and compare the enriched pathways (Fortino, 2015). (I)

Web-based tools EnrichR (Chen *et al.*, 2013) and ConsensusPathDB (Kamburov, Pentchev, Galicka, Wierling, Lehrach, & Herwig, 2011a) were utilized to examine the highlighted Gene ontology terms, and to predict gene interactions and functions through GeneMania (Warde-Farley *et al.*, 2010). (I)

Most significant genes and their close interactors were used for enrichment analysis, in order to understand the biological processes associated to different properties of CNM. Significant Gene Ontology terms were clustered with R package GOSemSim (Yu *et al.*, 2010). (II)

## 6.18 NETWORK INFERENCE (II)

Genes with high correlation to CNM properties were chosen for building networks by R-package MiNET (Meyer, Lafitte, & Bontempi, 2008). Median weight from each gene-pair was computed from all possible combinations of different inference algorithms, entropy estimators and discretization

methods, to find the best weighted adjacency matrix. Median weights together with Borda-method were then utilized for ranking the edges of the gene-pairs, in order to retrieve the most significant edges. For building interpretable subnetworks, with the most correlated, and co-expressed genes, different centrality measures were used: betweenness, clustering-coefficient, degree, closeness, and eigenvector. Genes were ranked according to correlation score and the centrality measures to retrieve the most significant genes from the networks. Genes from the subnetworks were further used to recognise the most enriched biological functions, utilizing gene ontology enrichment analysis with Fischer's exact test (Yu *et al.*, 2010).

# 7 RESULTS

## 7.1 OROPHARYNGEAL ASPIRATION TO RIGID, MULTI-WALLED CNT CAUSES ALLERGIC-LIKE RESPONSE IN MICE LUNGS (I)

To understand how lung responds to short time exposure to long and rigid multi-walled carbon nanotubes (rCNT), female C57BL/6 mice were exposed by oropharyngeal aspiration to 10 and 40 μg of rCNT dispersed in PBS, once per day for four consecutive days. Bronchoalveolar lavage (BAL) fluid was collected and cells were stained and counted. Both doses caused significant increase of granulocyte infiltration to the lungs as well as significant eosinophilic inflammation. Neutrophils were detected in both exposure setups as well. The strongest eosinophilic response was noted with the lower dose (10 μg rCNT per day), whereas higher neutrophil levels were observed after exposure to the higher dose of 40 μg rCNT per day. Macrophage counts were significantly lower with both doses when compared to the controls. Similarly, pathological evaluation of the lung tissues confirmed high eosinophilic response as well as accumulation of neutrophils and incomplete phagocytosis by macrophages (I, Figure 1b).

Cytokine IL-13 is known to orchestrate and enhance allergic response and asthmatic symptoms, by regulating eosinophilic inflammation, mucus secretion and airway hyperresponsiveness (Wynn, 2003). Since exposure to rCNT exacerbated high eosinophilic response, the expression of IL-13 was investigated in the lung tissue samples by RT-qPCR, and was found to be significantly expressed in lungs of the rCNT exposed animals with high and low dose (I, Figure 2d).

Mucin producing goblet cells, resident in the bronchial epithelial cell layer, are activated by signals mediated by IL-4 and IL-13. Goblet cell activation was distinguished based on the increased mucin production (PAS-positivity), after exposure to lower dose of rCNT, but seemed to have reduced activity with the higher dose of 40 μg per day (I, Figure 2f). These results confirm the harmful, allergic-type inflammation in the airways after 4-day exposure to rCNT and also suggest a dose-specific response, by changes in the activated leukocyte levels and mucin production after exposure with two different doses.

## 7.2 TRANSCRIPTIONAL RESPONSES ARE MIRRORING THE CELLULAR RESPONSES OF RCNT EXPOSURE IN MICE (I)

Transcriptomic responses were analyzed from the total RNA, extracted from lung tissues after oropharyngeal aspiration exposure to 10 μg of rCNT for four days (I).

Differentially expressed genes were obtained from quality checked, log2 transformed data, using empirical Bayes for pairwise comparisons (FC ≥ |1.5|, *post hoc* adjusted P-value 0.01). Gene expression levels of Th2-type cytokines IL-4 and IL-13, were found to be significantly upregulated in response to rCNT exposure. The expression levels of eosinophil chemoattractant genes CCL11 (eotaxin-1) and CCL24 (eotaxin-2) were also increased, confirming the activation of allergic-like response, mediated by cytokines IL-4 and IL-13 and eotaxins 1 and 2 (I, Figure 4).

To further characterize the biological responses to rCNT exposure, enrichment analysis was performed from the differentially expressed genes exploiting Gene Ontology terms and KEGG pathways. The sets of upregulated genes after rCNT exposure, were associated to gene ontology terms such as inflammatory response, cell cycle, chemotaxis and leukocyte migrations. KEGG pathway analysis highlighted pathways such as: receptor signalling, asthma, cytokine and chemokine signalling as well as metabolic pathways (I, Figure 5-7).

The enriched biological pathways related to allergy and inflammation support the observations on the cell-level responses and pathological evaluation, thus, advocating good concordance between cellular responses and transcriptional responses after 4-day exposure to rCNT.

## 7.3 OROPHARYNGEAL ASPIRATION CAN BE AN ALTERNATIVE METHOD TO INHALATION EXPOSURE IN MICE (I)

Oropharyngeal aspiration with the dose of 10 μg of rCNT per day for four consecutive days, was systematically compared to the previously performed inhalation exposure for 4 hours per day for four consecutive days by Rydman & Ilves *et al.* (Rydman *et al.*, 2014). Similarly to the inhalation exposure, the oropharyngeal aspiration to rCNT caused elevated levels of eosinophils, neutrophils and lymphocytes in the lungs as well as reduced levels of macrophages (I and II). Matching allergy-like, Th2-type response, with comparable magnitude of white blood cells was noted after inhalation exposure from BAL counts. Similar responses were also seen in abundant cytokine IL-13 secretion and in mucin production by goblet cell activation. From the differentially expressed genes, 56 % were common to the both exposure methods. Further on, both methods revealed high level (85 %) of

overlapping biological processes after Gene ontology enrichment analysis. Genes related to inflammatory response, receptor signalling and leukocyte migration were highly activated with the both methods. Overall, the results suggested high similarity in response to rCNT in mice lungs with both methods at cellular and transcriptional levels, suggesting, that oropharyngeal aspiration with carefully adjusted dose, could be used as a comparable method for inhalation.

## 7.4 CARBON NANOMATERIALS (CNM) CAUSE DISTINCT LEUKOCYTE ACTIVATION (II) IN MICE LUNGS

To investigate the immune cell activation in response to six carbon nanomaterials with different sizes and shapes, female C57BL/6 mice were exposed by oropharyngeal aspiration for four consecutive days with 10 μg per day (**Table 2** and **Figure 6**).

Following the exposures, BAL fluid was collected, and the leukocytes were stained and counted under light microscope. Distinct responses to different materials in leukocyte numbers were observed: rCNT elucidated significant increase in eosinophils, neutrophils and lymphocytes as concluded already from the results in the previous study (I). tCNT and Baytubes triggered mild increase in neutrophil counts. Macrophages were increased in response to fiber-like Graphite. Goblet cell activation through production of mucin was not significantly elevated with any material, but clear increase was noted after rCNT exposure (II, Figure S2).

Cellular responses evidenced material-specific responses, but only rCNT caused significantly different, and drastic reaction in the mouse lung, while exposures to other CNM triggered only mild responses in the cellular infiltration.

## 7.5 CNM PROPERTIES AFFECT TO THE TRANSCRIPTIONAL PATTERNS IN MICE AND IN THP-1 CELLS (II)

Six different carbon-based nanomaterials, with different shapes and sizes, were administered to female C57BL/6 mice for 4 days by oropharyngeal aspiration with dose of 10 μg per day and to PMA-differentiated macrophages, derived from human monocytic cell line (THP-1) with dose of 100 μg for 6 and 24 hours. Gene expression analyses were performed to study transcriptional patterns from both exposed organisms.

Gene expression data was hierarchically clustered to find similar transcriptional patterns between the CNM exposures. As expected, gene expression responses to rCNT formed a separate, specific cluster when

exposed to mice lungs for four days. Five other CNM, after *in vivo* exposure, clustered by their physical properties: spherical Fullerene and short SES, the materials with small aspect ratio clustered together, as well as materials with high surface area: Baytubes and tCNT. The only fibrous material in the series, graphite, segregated from the others. In the *in vitro* experiment, most of the materials clustered by the exposure time: 6 or 24 hours, but a separate cluster with rCNT 6h, SES 6h and tCNT 24h emerged, suggesting more specific response to these three materials (II, Figure S4).

## 7.6 CNM PROPERTIES: ASPECT RATIO, DIAMETER, LENGTH AND SURFACE AREA HAVE DISTINCT IMPACT TO *IN VITRO* AND *IN VIVO* EXPOSURES (II)

In order to clarify how distinct CNM properties affect the transcriptional responses, relationships between genes and CNM properties aspect ratio, diameter, length and surface area, were examined.

Correlation scores between the CNM property and the expression level of the orthologous genes from both *in vitro* and *in vivo* data sets, were calculated. This analysis evidenced distinct responses to CNM properties: Higher number of differentially expressed genes were correlating with length and aspect ratio *in vitro*, whereas *in vivo* exposures were correlating more with surface area and diameter, indicating method and organism specific responses to CNM exposures. As a conclusion, THP-1 cells seemed to be more responsive toward the length and aspect ratio, whereas mouse lung tissues were more sensitive to diameter and surface area (II, Figure 1).

## 7.7 TRANSCRIPTIONAL ANALYSIS OF DIFFERENTIALLY EXPRESSED GENES REVEALS NO COMMONALITIES BETWEEN *IN VITRO* AND *IN VIVO* EXPOSURES TO CNM PROPERTIES (II)

Orthologous genes from human and murine genomes were investigated by visualizing them in Venn-diagrams and pie charts (THP-1 6h, THP-1 24h and mice 96h.) Gene sets correlating to distinct CNM properties, varied from 50 to 639 genes per each exposure set up. The sets of expressed genes were not showing any overlap between the three separate time points (II, Figure 2), suggesting that *in vitro* and *in vivo* exposures have unique transcriptional responses to different CNM properties at different time points. Distinct sets of genes are activated in different organism and with different exposure method and time. This consequently demonstrates, that by simply studying transcriptional changes due to CNM exposures, differentially expressed genes cannot be utilized to disentangle common responses from *in vivo* and *in vitro* exposures.

## 7.8 NETWORK INFERENCE APPROACH CAN BE EXPLOITED IN DISCOVERING COMMON TRANSCRIPTIONAL RESPONSES TO DISTINCT CNM PROPERTIES IN MICE LUNGS AND HUMAN THP-1 CELL LINE (II)

The expression data from the above mentioned CNM exposures to THP-1 cells and mouse lungs was exploited to find common CNM property-specific biological responses within the exposure scenarios. Orthologous, differentially expressed genes from mice lung and human THP-1 cell line were retrieved from both data sets. Correlations to gene expression values and CNM properties were retrieved and several inference algorithms together with correlation scores and gene expression values, were utilized to build co-expressed and highly correlated genes. Several centrality measures were used to retrieve the most essential set of co-expressed gene sub-clusters, which were further utilized to find relevant biological responses to the CNM properties (II, Figure 3).

The approach revealed common biological functions, despite the exposure method or CNM properties (II, Figure 4). In addition to the common biological responses, also property-specific responses were found: Aspect ratio was activating biological processes related to angiogenesis, in all three exposure time points, in both exposure set-ups. Diameter-specific responses, NF-Kb activity and DNA repair, were found associated with *in vitro* and *in vivo* (**Figure 7**).

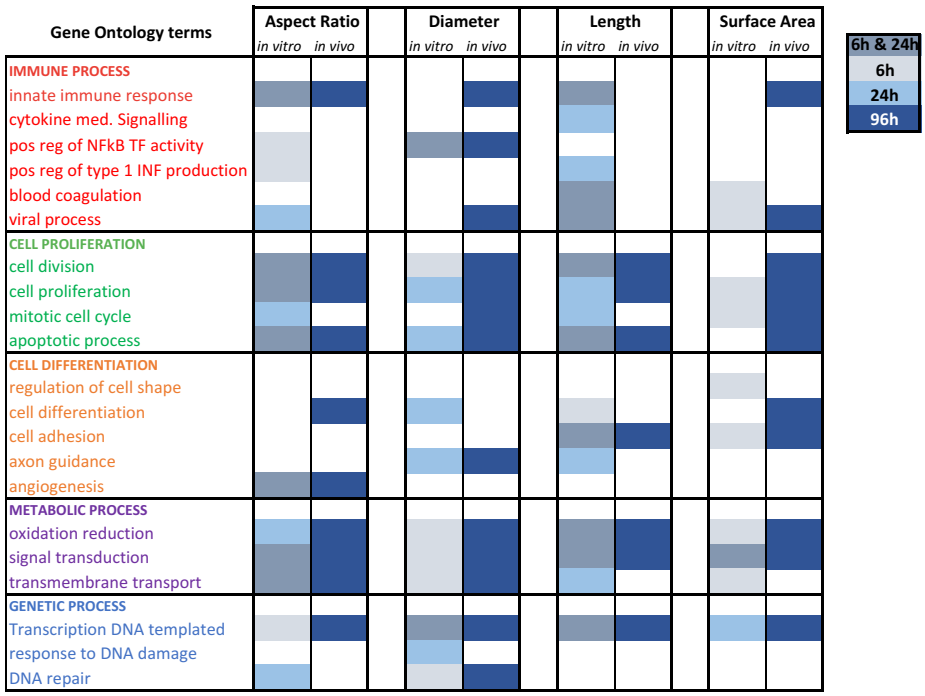


**Figure 7** Summary of the activated biological functions (Gene ontology terms) in response to CNM properties.

Network inference approach provides a way to disentangle common responses between *in vitro* and *in vivo*. The approach also enables the extrapolation of relevant information from *in vitro* exposures, classically regained only from extensive *in vivo* exposures.

# 8 DISCUSSION

Nanomaterials are rapidly pervading our everyday lives. Their benefits are well recognized, but the challenge lies in their safety assessment. Because of the high physico-chemical variation in different nano structures and forms, classical hazard assessment is not feasible. Traditional toxicology approaches are primarily based on animal tests that are not in general applicable to test the safety of ENM. For this, both, information on safety risks of ENM and adequate tests are needed. As its best, the novel testing methods are rapid, effective and comprehensive, mimicking human situation enough, preferably utilizing *in vitro* models, together with computational predictive modelling. When enough data and information about the effects of ENM is available, also efforts aiming at developing new safe-by-design materials could be exploited. At the same time, focus should be put also on educating research community and industrial partners about relevant regulatory issues. In this way, common ENM testing strategies as well as high concordance between the studies could be achieved. *In vivo* tests cannot be replaced before enough exposure data and knowledge is gathered, and before ensuring that *in vitro* assays can be reliably used to predict *in vivo* outcomes and toxicity to humans.

Nano-bio interactions are mainly studied by *in vitro* and *in vivo* exposure methods. Outcomes of these studies can reveal toxic potential of some ENM, but the outcomes are still somewhat controversial, resulting from several experimental problems such as proper ENM dose, toxicity end-points, exposure routes, used cell lines and animals, material purity, and experimental design.

Current high-throughput screening methods generate vast amount of new data to be used in hazard and risk assessment. Novel computational methods are greatly needed to retrieve relevant outcomes and specific phenotypes from large data sets. Also, advanced methods to predict the relevant biological outcomes and possible toxicity to human are needed for hazard assessment.

If ENM could be appropriately grouped and classified based on their different properties such as size, shape and reactivity, as well as their biological effects, more precise testing strategies and predictions about their possible toxicity and interactions with biological entities could be predicted. This in turn would facilitate the development of rapid testing methods and building of AOPs for unknown materials. The vast number of unique combinations of ENM physico-chemical properties are currently preventing any proper grouping. On the other hand, implementation of computational strategies to find specific molecular patterns, would aid the hazard assessment, allowing proper classification and more accurate predictions of

the exposure outcomes. Lack of common, standardized testing strategies and exposure methods, hinders the toxicity testing of ENM.

In this thesis, two strategies are introduced to overcome some of the current limitations in ENM toxicity testing: First, a faster and simpler mouse airway exposure method, the oropharyngeal aspiration, to be used instead of inhalation method and second, a systems biology approach based on gene network inference, to find common molecular responses from *in vitro* and *in vivo* ENM exposures.

## 8.1 SURROGATE SYSTEMS OF HUMAN AIRWAY EXPOSURES TO ENM

Processing and manufacturing of ENM cause a potential hazard to workers. Currently, no sizable epidemiological data about human exposure to ENM are available. Thus, several methods aiming to mimic the human exposure have been used. The inhalation exposure method utilizes rodents, to mimic real-life exposure scenario for humans. Thus, it is considered as the state-of-the-art method for studying acute and chronic pulmonary exposure effects (Oberdörster, Castranova, Asgharian, & Sayre, 2015). Inhalation exposure has disadvantages: It is time consuming and requires expertise from several scientific fields. The method also requires vast amount of ENM for generating a homogenized aerosol. Moreover, the aerosol generation is not possible for all ENM, especially the costly ones such as gold nanoparticles or nanodiamonds. In addition to the technical challenges, it is difficult to estimate the actual dose of animals because they are freely breathing the aerosolized material in whole-body exposure chambers. Also, contamination of the fur is likely to occur, causing secondary ENM exposure through the skin and gastrointestinal tract, triggering additional effects.

Oropharyngeal aspiration, as well as intratracheal and intranasal instillation methods have been successfully used to study inflammatory responses in murine models (Lacher, Johnson, Jessop, Holian, & Migliaccio, 2010; Poulsen *et al.*, 2015; 2016; Poulsen *et al.*, 2013). Since the material is introduced into the airways as a liquid dispersion, the amount of ENM entering the lungs can be estimated. Also, the small amount of ENM needed enables testing of expensive materials, as well as materials that cannot be aerosolized. Aspiration and instillation methods are relatively simple and quick when compared to inhalation, but they have drawbacks: dispersed ENM might result in additional material agglomeration. The dispersion delivered as a bolus, may distribute in different manner than when inhaled in aerosol, thus causing distinct responses in lungs. Moreover, mice need to be anesthetized, leading to possible additional response to inhaled isoflurane anaesthetic, and thus causing possible bias to the exposure. Also, questions about additional bio-corona formation in dispersion have been raised.

In publication I, particle characterization by DLS did confirm that the rCNT dispersions are not staying stable in time, thus raising concerns whether agglomeration causes the material to behave or react in different manner than inhaled aerosolized material. To diminish the agglomeration effects, the suspensions were prepared prior to use, and thoroughly vortexed before every administration. Nevertheless, also aerosolized particles tend to form aggregates (Nichols *et al.*, 2002), thus reducing the possible differences between the methods. Even though bio-corona formation is thought to have an impact on the cellular response, we observed no marked differences in the leukocyte infiltration in alveolar lavage or in histological evaluation or in the main cytokine/chemokine activation between the inhalation and aspiration methods.

### 8.1.1 RIGID MULTI-WALLED CARBON NANOTUBE INDUCES TH2-TYPE ALLERGIC RESPONSE

Adverse effects of long and rigid multi-walled carbon nanotubes have already been demonstrated in several *in vivo* and *in vitro* studies (Palomäki *et al.*, 2011; Rydman *et al.*, 2014; Poulsen *et al.*, 2013). Animal exposures to rCNT have been shown to cause lung inflammation, fibrosis, and mesothelioma, whereas *in vitro* cell exposures have revealed genotoxic and cytotoxic effects (Asakura *et al.*, 2010).

Some ENM are known to cause genetic damage, but only rCNT (Mitsui-7) has been associated to carcinogenicity (Toyokuni, 2013). Diameter and length of the rCNT particles have been suggested as the main cause of tumor-promoting effect (Chen & Wang, 2016). DNA damage might be due to direct contact with the genetic material (direct primary genotoxicity *i.e.* genotoxicity from the nanoparticles themselves) or disturbance in cell division machinery, such as microtubule spindles or centromeres, resulting in unbalanced cell division. Indirect primary genotoxicity might happen through imbalance in metabolic processes, leading to excessive ROS production (Donaldson & Poland, 2012). Interestingly, in addition to identified genotoxic potential, rCNT triggers Th-2 type of response, inducing secretion of cytokines IL-4, IL-5, IL-13, and production of IgE antibodies, usually signs of allergic inflammation and asthma (Dong & Ma, 2016; Inoue *et al.*, 2009; Rydman *et al.*, 2014).

In publication I, rCNT was chosen as a test material, since their effects on animals and cells have been already studied in-depth. Despite the experimental differences between the two exposure methods, oropharyngeal aspiration exposure to rCNT for four days was mirroring the cellular and transcriptional responses of inhalation exposure.

We observed dose-related cellular alterations in response to rCNT exposures to C57BL/6 female (I). Both 10 and 40 μg doses, in fact, increased the influx of eosinophils, neutrophils and lymphocytes. The presence of eosinophils is usually an indication of allergic reaction or asthmatic response,

while in healthy lungs the eosinophils are usually absent (Simon *et al.*, 2010). Eosinophilic inflammation was evident in both doses, but the higher dose of 40 μg elucidated significantly lower level of eosinophil infiltration to lungs, and significantly higher level of neutrophils when compared to lower dose of 10 μg per day. These results complement the outcomes of the previous studies with rCNT, and further suggest, that the higher dose of 40 μg per day shifts the response in airways towards acute lung injury, probably due to higher volume of rCNT aggregates.

The observed dose-dependency in response to rCNT is in concordance with results by Poulsen *et al.,* where similar decrease in eosinophilic response was also observed when the dose was raised from 18, 54 and to 162 μg/animal by intratracheal instillation. Decrease in macrophage counts with all three doses was also reported (Poulsen *et al.*, 2013). Due to similar cellular profiles in inhalation and lower dose aspiration, 10 μg aspiration dose was selected to study the transcriptional changes in publications I and II. In addition, activation of goblet cells, triggered by cytokines IL-4 and IL-13 further demonstrate Th2-type inflammatory response and support the concordance between both exposure methods.

In publication I, the analysis of the transcriptome responses to rCNT, highlighted pathways and biological processes that were in concordance with BAL cell counts and the histological evaluation: Pathways related to asthma, inflammatory responses, chemokine and cytokine signalling and activation of NOD- and TOLL-like receptor signalling suggest, that the transcriptomic patterns and activated biological processes can be utilized in understanding and predicting the cellular responses (I, Figure 5 and 6). Concordance in transcriptional changes and inflammatory cell influx in response to rCNT exposure, encourages the utilization of DNA microarrays to achieve deeper understanding of the cellular and molecular responses also in respect to other types of ENM.

### 8.1.2 CYTOKINES IN EOSINOPHILIC INFLAMMATION

Cytokine interleukin 13 (IL-13) is a central mediator of asthmatic response in lungs. It orchestrates eosinophilic inflammation, mucus secretion and airway hyperresponsiveness (Wynn, 2003). After one-week of rCNT exposures by inhalation and aspiration, expression of IL-13 cytokine was significantly increased in mouse lungs, when analysed with RT-qPCR and microarrays. Elevated levels of IL-13 corroborate the activated eosinophilic response. Also, the chemoattractants eotaxin-1 and eotaxin-2, which are recruiting eosinophils to the site of inflammation, were significantly elevated after rCNT exposure with the lower dose of 10 μg, by oropharyngeal aspiration. In addition, IL-4, another allergy and asthma related Th2 cytokine, with 70 % sequence homology to IL-13, was found to be elevated in similar manner with both methods (Hamid & Tulic, 2009; Schleimer *et al.*, 1992).

Th2 lymphocytes, the main producers of IL-13, are part of adaptive immune system, thus activated generally 96 hours after the exposure (Wynn, 2003). Thus, without immunological memory to rCNT, it is unlikely that Th2 lymphocytes are the main responders to rCNT exposure. It is hypothesized that the Th2-response might happen through airway epithelial cells, that are releasing "alarmin" signals such as IL-33 (Wang *et al.*, 2013). IL-33, in turn, is able to activate mast cells, macrophages, basophils and innate lymphoid cells, through ST2 receptor on the surface of the immune cells (Johnston & Bryce, 2017; Yagami *et al.*, 2010). Activated mast cells are then able to release the same cytokines as Th2 lymphocytes: IL-4, IL-5 and IL-13 (Smith, 2010). rCNT is characterized as long, rigid fibrous-like tube, thus, it is likely that the epithelial cell activation to release IL-33 might be due to mechanical irritation with direct nano-cell contact (Enoksson *et al.*, 2011).

### 8.1.3 ANIMALS AND CELL LINES

Genetically stable, C57BL/6 mouse strain was used in publications I and II. Inbred strain provides homogenous genetic background, enabling reliable and comparable study set up in search of specific transcriptome-derived signatures.

For the same reason, as well as for them being human-derived, monocytic cell line was utilized in publication II. THP-1 cell line provides higher homogeneity compared to individual donor cells. Macrophages are the first sentinels of the lung immune system, responding quickly to the invasion of foreign materials.

The growing number of ENM makes the testing of all individual nanomaterials unmanageable with the current *in vivo* exposure methods. Testing the toxicity of all ENM individually should cover all the relevant exposure routes, doses and exposure times, posing a severe drawback for the nanomaterial hazard assessment. As stated earlier, inhalation method is laborious and time consuming, and alternative pulmonary exposure methods need to be developed. Even though oropharyngeal aspiration does not respond to the current need for rapid toxicity testing alone, it is providing a simplified and faster way to examine the ENM effects on airways. Data about human exposures to ENM is not available, making animal and cell models the only way to gain more knowledge and to evaluate the effects of ENM exposure to human. In study II, transcriptional responses of human PMA-differentiated macrophages and mouse airways were compared. Transcriptional patterns and gene activation can vary between species (Heinonen, 2015). Expectedly, this was also concluded when the activated set of ortholog genes were compared. Nevertheless, similar biological responses were noted between the two species when the network approach was utilized.

## 8.2 SYSTEMS TOXICOLOGY APPROACHES FOR ENM TOXICITY STUDIES

Studying the molecular alterations provides the possibility to gain significant knowledge about the ENM mechanisms of actions (MOA). For instance, the investigation of the transcriptomic alterations after exposure to nanomaterials is also thought to provide broader understanding of the mechanisms underlying ENM toxicity (Nel, 2013; Nel *et al.*, 2013; Sturla *et al.*, 2014). In this respect, transcriptional mechanisms of action (TMOA) provides an important layer of information to complete adverse outcome pathways (AOPs), and thus, to reach more comprehensive conclusions about toxic potential of ENM.

Transcriptomic profiling usually creates overwhelming amount of data. The challenge lies in the interpretation of the biological meaning behind the data sets. As recognized in publication II, gene expression profiling *per se* is not providing systematic information that can be utilized in interpreting complex patterns of molecular interactions between different species and between *in vivo* and *in vitro* exposure methods. Different sets of genes were altered in response to distinct exposure scenarios at different time points. In addition, expression patterns in THP-1 cell exposures were mainly associated to the time of exposure (II, Figure S4). Thus, different computational approaches are needed to mine the datasets for discovering the most relevant communities of systematically interacting molecules, and further to gain understanding of the important biological processes affected by different ENM and their properties.

Several computational tools have been developed to fill the knowledge gaps in classical hazard assessment. QSAR, read-across and adverse outcome pathways are utilized in hazard assessment when possible, to draw more comprehensive picture about the mode of action of the substance.

Results in the publication I offer valuable information about the similarity of the exposure methods in mice, thus providing easier and cost-effective way (oropharyngeal aspiration) compared to inhalation method to test toxic effects of nanomaterials after pulmonary exposure. Transcriptional profiling is further complementing the cellular outcomes and providing more information about the molecular responses. When more data about the molecular signatures in respect to exposure to distinct nanomaterials is gathered, new promising hazard assessment methods could be successfully utilized also in ENM toxicity testing and risk assessment.

### 8.2.1 TRANSCRIPTIONAL ANALYSIS OF ENM EXPOSURES

Transcriptome analysis is widely used method to characterize molecular alterations in response to diseases and other physiological and pathological conditions. To date, transcriptome-derived signatures are exploited to perform differential diagnosis of many human diseases, predict the most

effective drug treatments (Dopazo, 2014; Lamb *et al.*, 2006) and characterize the effects of chemical exposures in toxicology studies (Bourdon *et al.*, 2013; Li *et al.*, 2016). More and more transcriptome analyses are also used in respect to ENM exposure (Eom *et al.*, 2015; Jennings, 2013; Labib *et al.*, 2016; Marx-Stoelting *et al.*, 2015; Nel, 2013; Poulsen *et al.*, 2015; Robinson *et al.*, 2012; Poulsen *et al.*, 2013). With gene expression profiling and computational approaches, a greater depth of understanding about the ENM responses could be achieved.

Transcriptional responses from different study groups are often compared from the lists of differentially expressed genes or activated pathways. This strategy was indeed an effective approach in publication I, where the two exposure methods were compared by using same exposure time and correlating doses within the same organism (mice). In publication II, the different properties of CNM activated distinct sets of genes in mouse lungs *in vivo* and human macrophages *in vitro*, suggesting no correlation between *in vitro* and *in vivo* results (II, Figure 2). This was indeed anticipated, since the complex interactions between several cell types in mice lungs, compared to homogenized THP-1 cell line. In the scenario, where different species and exposure methods are compared, more systematic analysis, considering the complex patterns of molecular interactions, need to be employed.

### 8.2.2 INFERENCE OF GENE NETWORKS RESPONDING TO ENM EXPOSURE

Systems toxicology approach cannot replace the regulatory toxicity testing strategies, but is aiding the shift from canonical toxicity testing towards predictive, and safe-by-design approaches. This applies not only to nanomaterial testing but to regulatory toxicology in general. When ENM properties affecting to toxicity mechanisms and other effects are sufficiently recognized, *in vitro* studies together with computational approaches could be applied in greater amounts to search for the relevant responses defining the toxic potential of ENM to humans and environment.

Genes do not function alone, but through complex patterns of synergistic co-operation. Consequently, inference of co-expression gene networks, can highlight specific programs of transcriptional regulation. When the signatures of expressed genes are studied in respect to intrinsic properties of ENM, specific transcriptional responses of co-regulated genes to distinct ENM properties can be emphasized. Recognizing the nanomaterial structures and properties leading to adverse outcomes is an important step towards predictive toxicology approach and utilization of strategies such as AOP or QSAR. For this reason, in publication II, gene expression data was used to systematically infer co-expression networks emerging in response to specific ENM properties. Network inference with several centrality measures,

highlighted more specific gene sub-clusters, found to represent significant biological processes across different exposure scenarios (II, Figure 4).

## 8.3 TOWARDS ENM PROPERTY-BASED HAZARD ASSESSMENT

Shape and size of ENM have an effect to the cellular and transcriptional responses and possible toxicity outcome (Albanese, Tang, & Chan, 2012; Braakhuis, Park, Gosens, de Jong, & Cassee, 2014; Poulsen *et al.*, 2015; Sohaebuddin, Thevenot, Baker, Eaton, & Tang, 2010). Since the high heterogeneity of different ENM properties and their combinations, the prediction of the toxic outcomes is not practically possible with the present tests. More relevant and high throughput methods would enable the screening of more materials and to gain more data for computational modelling and hazard predictions.

All the six tested CNM, were considered as pure carbon materials (pure carbon content > 99%). Based on the assumption on chemical similarity between the tested CNM, it was hypothesized, that the distinct responses to lungs and cells are likely to be caused by shape of the CNM. Shape might also have an impact on the agglomeration status, but is unlikely, that agglomeration would explain all the cellular and molecular differences, as pointed out in publication I.

In study II, BAL cell counts demonstrated different cellular responses to the six distinct CNM in mice. Only rCNT activated clear eosinophilic response, while Baytubes and tCNT induced slight neutrophilic inflammation. Based on BAL counts, one could conclude, that most of the CNM are either rapidly cleared from the airways, nor immune system does not recognize them as foreign. However, microscopic evaluation evidenced particle penetration and retention in lungs after four-day exposure. Based on this, the second postulation seems more probable. Since BAL cell counts evidenced clear inflammatory response only to rCNT, more in-depth analysis is needed to draw comprehensive conclusions of the specific responses and transcriptional alterations in response to CNM and their properties.

The well recognized fibre paradigm highlighting the high-aspect ratio nanomaterials (HARN) is holding true, but is mainly associated to the length and rigidity of the material (Donaldson *et al.*, 2010; Nagai *et al.*, 2011). In addition to the length and high aspect ratio, also surface area and diameter of the CNM have an effect to transcriptional responses. In publication II, examination of the gene expression patterns correlating to CNM properties, revealed different expression patterns in *in vitro* and *in vivo*.

Indeed, the results in publication II clearly indicated that molecular responses are affected by the shape of the particle. Correlation distributions of the gene expression patterns were different between the CNM properties:

Aspect ratio and length had a stronger impact on the cells exposed *in vitro*, whereas diameter and surface area correlated more with *in vivo* exposures (II, Figure 1). What causes the genes expression patterns in THP-1 cells to correlate more with aspect ratio and length, and mouse lung to correlate with diameter and surface area, remains still unsolved. However, it is possible to hypothesize that different material accumulation and sedimentation in different exposure set ups have an impact, along with specific regulation of cellular uptake mechanisms. Heterogeneity of the mouse lung cell population is understandably also affecting these processes.

### 8.3.1 CNM PROPERTIES PROMOTE SPECIFIC BIOLOGICAL RESPONSES WITH NETWORK-BASED APPROACH

As stated earlier, shape of the ENM influences the cellular fate and how the particle is recognized in biological environment.

Indeed, in publication II, biological processes were highly correlated to different CNM properties (**Figure 7**). For example, genes involved with angiogenesis are correlating with CNM aspect ratio both *in vivo* and *in vitro*. Angiogenesis has gained attention in several nanomaterial related studies. Anti-angiogenetic potential of some ENM has been studied as a treatment to tumors, whereas pro-angiogenesis has been studied in respect to vascular engineering. For example, Han *et al.* studied nanofibrous scaffold of MWCNT, and found elevated endothelial cell proliferation and aggregation along the nanofibers (Dobrovolskaia & McNeil, 2013; Han *et al.*, 2009).

The expression of genes involved in the response to DNA damage and DNA repair, on the other hand, were found to correlate with the particle diameter. Sharp edges and particles with small diameter are capable to escape the endosomes after cellular uptake, and thus lead to intracellular translocation and possible interaction with DNA. Chu *et al.*, demonstrated that the particle morphology has a dominant role in cellular fate (Chu *et al.*, 2014; Nagai *et al.*, 2011). DNA damage can also arise through ROS generation, presumably *via* lysosomal membrane destabilization, causing lysosomal content leaking into the cytoplasm. Sohaebuddin *et al.* studied ROS production in three different cell lines, with three MWCNT differing by diameters. The diameter affected to the ROS production in all cell lines, as the highest degree of ROS was measured with MWCNT with smallest diameter (< 8 nm) (Sohaebuddin *et al.*, 2010).

NF-Kb activation pathways were also associated to diameter *in vitro* and *in vivo*. Indeed, ROS can activate NF-**κ**b transcription factor, inducing in turn the pro-inflammatory cascade and apoptosis (Hussain *et al.*, 2014). Deng *et al.* suggested that, in addition to the known ROS-stimulated pro-inflammatory activation by ENM, also other properties of ENM can induce pro-inflammatory effects. They demonstrated NF-Kb signalling pathway initiation with 5nm gold nanoparticles, but not with 20nm particles (Deng, Liang, Monteiro, Toth, & Minchin, 2011).

Publication II indicates that diameter and length indeed have some exclusive effects, activating distinct biological processes. The results further suggest that, when properly assessed, similar biological responses can be highlighted both in mouse lungs and in THP-1 cell line.

Interestingly, surface area did not reveal remarkable specific responses as compared to the other particle properties analysed, involving genes associated mostly to generic processes. Common cellular responses such as signal transduction, cell proliferation and transcription were expectedly associated to all CNM properties in both organisms.

The transcriptional responses of one cell line cannot completely reflect the complex interactions and reactions occurring between multiple cell types in the airways. Nevertheless, since the activated biological responses are partly overlapping in mice *in vivo* and human THP-1 cells *in vitro*, and with three time points (II, Figure 4), it could be envisioned that ENM exposures to *in vitro* co-cultures or 3D-models could provide more inclusive concordance to *in vivo* responses. Since testing of all possible materials with *in vivo* exposure methods seem to be out of reach, it is understandable that cell models, or organ-based models together with systems toxicology approaches could be a good alternative and thus there is a need for this strategy to be further developed and improved for risk and exposure assessment. In the current comparison, also the doses vary from *in vitro* dose of 100 μg and *in vivo* dose of four times 10 μg. A better dose correlation would most probably also improve the concordance. When standardized *in vitro* approaches become available and more common, high-throughput screenings and computational methods would enable more robust risk assessment methods first, by improving the understanding of the biological responses related to distinct ENM, second, by simplifying exposure assessments and reduction of animal models, third, by predicting exposure outcomes in human through *in vitro* based methods, and fourth, by building predictive models for hazard assessment and facilitating the production safe-by-design materials.

# 9 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

New methods to assess nanomaterial toxicity are needed to guarantee their safety while ensuring innovation. Sizable ENM exposure data are being produced, but the lack of concordance between the study setups represents a bottleneck, preventing comprehensive conclusions in understanding nanomaterial behaviour. In addition, mouse models, commonly used in traditional risk assessment, may not be optimal to study hazardous potential of ENM to humans. In this thesis, new perspectives to more robust nanotoxicology approaches are provided:

Severely toxic rigid multi-walled carbon nanotube (rCNT), causing allergic-type, eosinophilic inflammation to mouse airways, was shown to cause comparable inflammatory response in mouse lungs with oropharyngeal aspiration and inhalation exposure methods.

The results suggest that oropharyngeal aspiration, a simpler *in vivo* exposure method, can be utilized to gain knowledge about the ENM toxicity. When easier exposure methods are used, more data can be collected in more effective and quicker manner.

In addition to rCNT, transcriptional alterations of five other carbon nanomaterials (CNM) were studied in an *in vitro* model of human macrophages and mouse lung tissues *in vivo*.

The observations of this second study indicate that transcriptomics experiments can successfully recapitulate the transcriptional mechanisms of action of CNM. More importantly, a novel analytical approach based on the inference of gene networks, but not traditional univariate data mining strategy, is able to highlight significant overlap between the molecular alterations *in vitro* and *in vivo* consequent to CNM exposure. These results pave the way to a deeper implementation of transcriptomics technologies and *in vitro* models to the safety assessment of ENM.

Several large research projects in the area of nanosafety have been carried out to date. The lack of standardized testing methods complicates interpretation of the results and data integration. For this, validated exposure settings, together with high throughput screenings, are needed for systems toxicology-based hazard assessment.

Adverse outcome pathway (AOP) can be used to efficiently gather and link mechanistic data and relevant end points needed for the hazard assessment (LaLone *et al.*, 2017). AOP consists of structural presentation of initiating effects and ordered series of key-events leading to adverse outcome providing comprehensive picture about the MoA (**Figure 5**). Systems biology

approaches, such as those presented in this thesis, could provide more detailed representations of the molecular key events that lead to adverse outcomes. Even though the studies presented here focused on transcriptional changes, the analytical strategies employed are scalable to any big data sets to be mined in search of molecular signatures and key events leading to adverse outcome.

When enough standardized ENM exposure data are available, better and more robust computational approaches could be applied to develop AOP and QSAR models, ultimately leading to predictive toxicology approaches, benefitting regulators, toxicologists, industry and, ultimately, society.

To replace laboratory animals in toxicity tests, more sophisticated, high quality *in vitro* approaches should be developed and their relevance to mimic human situation should be proved, thus, finding ways to simplify risk assessment, simultaneously reducing use of laboratory animals. The current study considers 4 intrinsic properties of carbon nanomaterials, the aspect ratio, length, diameter and surface area. To utilize the approach to different nanomaterials, also other relevant ENM properties should be considered, such as core chemistry, charge and functionalization. When detailed information on ENM structure-activity relationships are determined, also specific nanomaterial designs can be used to produce next-generation nanomaterials. This thesis focuses solely on carbon-based nanomaterials, and should be taken into account, when examining the toxic effects of other ENM, such as metal-based and synthetic nanomaterials. Characterized, reference materials are needed to ensure high concordance between the obtained results. Also, different doses and time points should be examined for more in-depth comparisons.

This work provides insights into systems toxicology based hazard assessment. Although ENM doses, exposure times, cell types and animal models still need to be studied to gain inclusive insights from the biological responses, this thesis contributes to develop simpler, non-animal-based nanomaterial toxicity testing and systems toxicology approaches.

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