Toxicity and biological impact of metal and metal oxide nanoparticles

Focus on vascular toxicity of ultra-small titanium dioxide nanoparticles

Narges Bayat
Cover: A hyperspectral image of the heart of a zebrafish embryo with TiO$_2$ ultra-small nanoparticles (identified by the red spots). The exposure was performed at 0 hour post fertilization (hpf) through injection and the images was taken at 120 hpf by Cytoviva microscopy.

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Abstract

The growing production and application of nanoparticles (NPs) in different technologies has led to tremendous advancement in those fields. Moreover, there is currently interest in using ultra-small NPs (USNPs) at 1-3 nm, due to their unique large molecule like features. Consequently, detailed assessment of the safety of NPs and USNPs is necessary. To this end, the main goal of this thesis was to investigate the toxicity and underlying mechanisms following exposure to different metal and metal oxide NPs as well as USNPs. Their effects were studied on *Saccharomyces cerevisiae*, on hepatocytes and endothelial cells and finally *in vivo* on zebrafish embryos (*Danio rerio*). By selecting the rutile form of titanium dioxide (TiO$_2$-USNPs) without intrinsic or intracellular reactive oxygen species (ROS) production, we could study biological impacts solely due to size and direct interaction with the cells. We showed that TiO$_2$-USNPs were not cytotoxic but induced DNA damage. They had anti-angiogenic effects both *in vitro* and *in vivo*. Also, at high concentrations they caused complete mortality in zebrafish embryos exposed in water, while at lower concentrations, they induced delay in hatching. When injected they caused malformations. They specifically induced the differential overexpression of transcripts involved in lipid and cholesterol metabolism in endothelial cells. In hepatocytes they induced the overexpression of proteins in the electron transport chain and decreased lipids in lipid rafts. At 30 nm, TiO$_2$-NPs, were also genotoxic, but they were not cytotoxic and had no effects *in vivo* or *in vivo* on angiogenesis. However, they induced differential expression of transcripts involved in endoplasmic reticulum (ER) stress and heat shock response as well as cholesterol metabolism. This suggests a more toxic response in the cells compared to TiO$_2$-USNPs. Single walled carbon nanotubes (SWCNTs), despite having the highest oxidative activity among the NPs studied, were not severely cyto- or genotoxic, but induced expression of transcripts involved in early ER stress response. Copper oxide NPs (CuO-NPs) exhibited highest toxicity due to both ion release and ROS production, affecting lipid metabolism of the cells. Silver NPs (Ag-NPs) were also cytotoxic and caused the disruption of cellular components and lipids. Zinc Oxide NPs (ZnO-NPs) were not cytotoxic but they increased the size of vacuoles in yeast cells. Finally, by using superparamagnetic iron oxide NPs (SPIONs) with different coatings, and using a mathematical model, a nano impact index (INI) was developed as a tool to enable the comparison of nanotoxicology data.
List of publications


IV. Bayat N, Cristobal S. The effects of ultra-small TiO$_2$ nanoparticle and single walled carbon nanotubes on endothelial cells: next generation sequencing and transcriptome sequencing (RNA-seq) analysis. [Manuscript]


*These authors contributed equally
Additional publications

VI. Lopes V.R, Loito V, Audinot J-N, Bayat N, Gutleb A.C, Cristobal S. Dose effect of TiO$_2$-NPs elicits autophagy modulation in skin cells: two sides of the same coin [manuscript under review]
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<td>2D-DIGE</td>
<td>Two dimensional difference gel electrophoresis</td>
</tr>
<tr>
<td>AOP</td>
<td>Adverse outcome pathway</td>
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<tr>
<td>BME</td>
<td>Basement membrane extract</td>
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<tr>
<td>CyDye</td>
<td>Cyanine fluorescent dyes</td>
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<td>DCFH-DA</td>
<td>2’, 7’ dichlorofluorescein diacetate</td>
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<td>DLS</td>
<td>Dynamic light scattering</td>
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<td>HMEC-1</td>
<td>Human dermal microvascular endothelial cells</td>
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<td>ICP-MS</td>
<td>Inductively coupled plasma mass spectrometry</td>
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<tr>
<td>INI</td>
<td>Integrated nanoimpact index</td>
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<tr>
<td>LD</td>
<td>Lipid droplet</td>
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<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
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<tr>
<td>MTT</td>
<td>3-(4, 5- dimethyl diazo-2-yl)-2, 5 diphenyltetrazolium bromide</td>
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<td>NGS</td>
<td>Next generation sequencing</td>
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<td>nLC-ESI-MS/MS</td>
<td>Nano-HPLC electrospray ionization multistage tandem mass spectrometry</td>
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<td>NPs</td>
<td>Nanoparticles</td>
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<tr>
<td>OTM</td>
<td>Olive tail moment</td>
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<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
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<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>SM</td>
<td>Sphingomyelin</td>
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<td>SPIONs</td>
<td>Super paramagnetic iron oxide nanoparticles</td>
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<td>SWCNT</td>
<td>Single walled carbon nanotubes</td>
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<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TG</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
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<tr>
<td>TMRE</td>
<td>Tetramethylrhodamine, ethyl ester</td>
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<tr>
<td>USNPs</td>
<td>Ultra-small nanoparticles</td>
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1. Introduction

The term “nano” is derived from the Greek word meaning dwarf and is used to represent one billionth \((10^{-9})\) of a meter. Nanoparticles (NPs) are defined as particles of matter with at least one dimension from 1 to 100 nm. At this size range they exhibit distinct physicochemical properties compared to larger particles with the same constituents such as different optical, electrical and catalytic activity\(^1,2\). These unique features of NPs make them desirable to be used in many applications such as cosmetics, paints, textile and food packaging.

According to historical studies, the intentional application of NPs by humans dates back to as early as AD300, as evident in Damascus steel blades. Scientists have discovered that these swords, known for their incredible strength, shatter resistance and exceptional sharpness, contain a certain type of NPs known as carbon nanotubes\(^3\).

NPs with natural origins such as forest fires and volcano eruptions have always existed in the environment\(^4\). Due to the advent of nanotechnology, however, different types of NPs are rapidly produced and commercialized. According to a statistical study, the worldwide production of TiO\(_2\)-NPs is estimated at 10 000 tons/year between 2011–2014 and expected to reach 2.5 million tons/year by 2025\(^5\).

Despite the great advantages of nanotechnology, since the size of NPs is comparable to the typical cellular machinery and proteins, there is therefore concern regarding their potential disturbance of living cells and adverse effect on human health. There is growing evidence that NPs can cause damage and diseases at their exposure site, such as lungs or skin. They may also enter the circulatory system where they can cause disturbances that could lead to cardiovascular diseases such as atherosclerosis and high blood pressure. Moreover, through the circulatory system, they might translocate to other organs such as the liver and cause disease there.

Therefore, in 2004 the multidisciplinary field of Nanotoxicology was officially introduced with the purpose of characterizing and studying the biological impact of NPs in support of a safe and sustainable nanotechnology\(^6\). In the last decade the interaction between NPs and biological systems has been explored, but the toxicity of the smallest NPs has attracted very little attention. These so called USNPs which are between 1 and 3 nm could open new possibilities. Since at this size range they exist in between complete molecular dispersions and larger-sized NPs and thus exhibit intermedi-
ate physicochemical properties. So far, different USNPs have been examined for use in medical technologies as well as for the reduction of the toxicity of inorganic particles\textsuperscript{7,8}.

The main purpose of this thesis is the study of the toxicity and biological impact of some of the most widely used NPs and USNPs on the liver and the cardiovascular system.

I begin this thesis by describing NPs and USNPs and why their physicochemical properties are unique, followed by presenting the NPs investigated. Then, since the cardiovascular system is an integral route of exposure to NPs, I argue in chapter 3 how the NPs enter and impact different aspects of this organ. In chapter 4, I discuss how the physicochemical properties of NPs affect their toxicity and biological impact. Then the methods used and a short summary of the studies conducted will be presented in chapter 5 and 6. Finally, a short discussion on our most important findings as well as some of the challenges encountered when performing nanotoxicological studies and how they might be circumvented is presented.
2. Nanoparticles

2.1 Physicochemical properties

The unique properties of NPs are strongly related to the exponential increase in the ratio of the number of surface atoms of the particle, to the total number of atoms, as the size decreases\textsuperscript{9,10}. The increase in the surface area to volume ratio contributes to “surface effects” in NPs. Because the atoms situated at the surface have fewer neighbors than bulk atoms there is subsequently lower binding energy per atom. This thermodynamic instability in turn impacts the interfacial reactivity and thereby intrinsic properties of NPs such as their dissolution and melting point. For example, the melting temperature of indium NPs is reduced by 120 °C when the size is decreased from 100 to 10 nm\textsuperscript{11-13}.

Other important characteristics of NPs are their oxidative activity and superparamagnetism. Firstly, due to the size-dependent differences in atomic and electronic structure, active sites are formed on the surface of NPs that where the reactive oxygen species (ROS) can be produced. The NPs can thus gain affinity for electron uptake and subsequent transfer to species in solution\textsuperscript{11,14}. One method for producing ROS is through photocatalysis (Figure 1). The activation of TiO\textsubscript{2}-NPs by UV light generates electrons and holes in the conduction and valence bands, respectively. The photo-generated charges take part in reduction and oxidation reactions at the NPs surface\textsuperscript{15}. These reactions can lead to the production of ROS (e.g. O\textsubscript{2}+e\textsuperscript{-}→O\textsubscript{2}^{-}) or oxidation of organic compounds\textsuperscript{16}.

![Figure 1. Photocatalysis in TiO\textsubscript{2}-NPs.](image-url)
Secondly, superparamagnetism is exhibited by magnetic nanoparticles. Iron oxide ($\text{Fe}_3\text{O}_4$) NPs at $< 20$ nm contain only one magnetic domain in contrast to multiple domains in larger particles. Therefore their magnetization directions are subject to thermal fluctuations, and without an external magnetic field, their net magnetization value is zero. Upon application of an external magnetic field, the NPs align with the field direction, obtaining magnetic saturation at high magnitudes\textsuperscript{17,18}.

The physicochemical properties of NPs are not only size dependent but are also affected by factors such as their constituents and crystal structure. These parameters can therefore be used to classify NPs. For the purpose of this thesis we classify them based on type. (Figure 2)

![Figure 2. Classification of the type of engineered NPs](image)

### 2.2 USNPs

The properties of NPs change when they are in the 1-3 nm range because they have an extremely large surface area to volume ratio\textsuperscript{19, 20}. Therefore most of the constituting atoms reside at the surface enhancing their reactivity\textsuperscript{21,22}. As shown in figure 3, the percentage of atoms on the surface of 1 nm USNPs is about 80% , they essentially have no true core\textsuperscript{23, 24}.
Figure 3. The percentage of surface atoms in relation to particles diameter. Image with permission from Nützenadel et.al\textsuperscript{23}

Moreover, since the USNPs only contain several to about 500 atoms even minor atomic changes in size can lead to dramatic differences in their properties. For example, although the difference between Au\textsubscript{38} and Au\textsubscript{40} USNPs is only two atoms, they exhibit different Lewis acid properties\textsuperscript{25}. In addition, the different quantum states affect the properties of USNPs. Ag-USNPs become fluorescent, while Au-USNPs exhibit different crystal structure (Au-NPs are face-centered-cubic (FCC) while Au-USNPs are non-FCC), and have enhanced catalytic properties\textsuperscript{21,26,27}. These unique features offer new possibilities for their application and enhancement of technologies such as drug carriers or catalysts. In order to realize this potential however, monodisperse size distributions, appropriate characterization techniques as well as understanding their interaction with biological systems is necessary.

\textbf{2.3 NPs investigated}

\textbf{TiO\textsubscript{2}-NPs}

Titanium is the ninth most abundant element in the earth's crust (4400 mg/kg), but due to its great affinity for oxygen and other elements, it does not exist in the metallic state in nature\textsuperscript{28}. TiO\textsubscript{2} particles naturally occur in three crystalline forms i.e. anatase, rutile, and brookite (Figure 4). Rutile is the most common and chemically stable of the three. Anatase, on the other hand, is the most reactive and can produce ROS\textsuperscript{29,30}. 
Another property of TiO$_2$-NPs is water splitting. The water oxidation reaction can be written as $\text{H}_2\text{O} + \text{hole}^+ \rightarrow 1/2\text{O}_2 + 2\text{H}^+$. A recent study has shown that rutile surface is more active for water splitting kinetically compared to anatase. This was however, not due to the redox level of the hole (the position of the valence band maximum), but rather due to the more favorable local bonding geometry of the surface, which reduces the barrier of the O–H bond breaking.

Due to the photocatalytic property, the NPs (as illustrated in Figure 1) are used for applications such as removal of air pollutants or as disinfectants. Also TiO$_2$-NPs are thus one of the most highly used NPs in industrial and environmental applications such as paints and sunscreens.

Most of the toxicity observed for TiO$_2$-NPs exposure is attributed to anatase and oxidative stress. The toxicity of rutile and brookite on the other hand is not as fully understood. Therefore investigation of the biological impact of TiO$_2$-NPs without oxidative activity could provide an insight to the safety aspects of their use in industry.

Moreover, due to their insoluble nature and properties described above, TiO$_2$-USNPs could be potentially used for medical applications. In the few studies on their effect in vivo however, it was shown that TiO$_2$-USNPs could induce lung damage and changes the permeability of alveolar-capillary barrier. In this particular case the TiO$_2$-USNPs had caused oxidative stress. Therefore, we studied the effects of rutile TiO$_2$-USNPs and TiO$_2$-NPs without intrinsic oxidative activity (paper I-IV).

**SWCNTs**

SWCNTs consists exclusively of carbon atoms arranged in condensed aromatic rings, organized in a single cylindrically folded sheet. The dimension of SWCNTs is 0.4–2.0 nm in diameter and a few μm in length. They have semiconducting activity depending on their chirality (i.e. the chiral angle between hexagons and the tube axis) with either large (~0.5 eV) or
small (∼10 meV) bandgaps\textsuperscript{38}. The chemical reactivity of SWCNTs is similar to that of olefins (i.e., C=C double bonds) therefore events such as electrophilic oxidations and free radical reactions including alkylation are possible.

![Image of SWCNTs inside intracellular vesicles](image_url)

**Figure 5.** SWCNTs inside intracellular vesicles in endothelial cells (paper III). The zoomed section is schematic presentation of the structure of SWCNTs.

SWCNTs have found increasing popularity due to their high tensile strength and light weight and are therefore used in electronics and are considered for novel approaches in drug delivery\textsuperscript{39}. Due to the high aspect ratio (i.e. ratio of the length to the diameter) of SWCNTs and multi-walled carbon nanotubes (MWCNTs), it has been considered whether they might illicit asbestos-like response in cells\textsuperscript{40}. Furthermore, their high oxidative activity could attenuate vasodilation\textsuperscript{41,42}.

On the other hand, several *in vitro* and *in vivo* experiments indicate that they are well tolerated\textsuperscript{43}. In order to address some of these questions we study the effect of SWCNTs on the cardiovascular system (paper I, III-IV).

**CuO-NPs**

CuO-NPs are semiconducting compounds with a monoclinic structure and are widely used as catalysts, in printed electronics, solar energy conversion and antimicrobial products\textsuperscript{44}. Studies have shown that CuO-NPs are highly toxic to prokaryotes and eukaryotes compared to other metal NPs\textsuperscript{20,45}. The divalent ion forms of Cu (and Zn) are essential for all living organisms as they are cofactors for a number of enzymes. Therefore the study of the solubility effect at low doses from CuO-NPs could provide an insight to the mechanism behind their toxicity (paper I, II).

**ZnO-NPs**

ZnO is characterized by having a photocatalytic and a photo-oxidizing effect on chemical and biological species\textsuperscript{46}. ZnO-NPs have stable wurtzite
structure and are wide bandgap (3.4 eV) semiconductors. They can be used as catalysts and in electronic devices as well as in cosmetics and modern sunscreens.\textsuperscript{47} The worldwide annual production of ZnO-NPs is estimated to be around 550 tons/year thus having the third highest global production volume after silicon dioxide and TiO\textsubscript{2}-NPs.\textsuperscript{48} The consensus regarding toxicity of ZnO-NPs is that it is strongly dependent on the exposure concentrations and their solubility. This means that while some studies shows that they are not toxic, others point to oxidative DNA damage and apoptosis.\textsuperscript{49} In vivo studies in general indicate that inhalation of ZnO-NPs can be toxic.\textsuperscript{50} As with CuO-NPs, the effect of low doses of ZnO-NPs on hepatocytes was studied in paper I-II.

**Ag-NPs**

Pure Ag has the highest electrical and thermal conductivity as well as the lowest contact resistance of any metal.\textsuperscript{51} Ag-NPs have been used for more than 100 years. Today they are one of the most commonly used engineered NPs in antimicrobial agents, food products as well as in medical applications.\textsuperscript{52,53} Approximately 320 tons of Ag-NPs are manufactured annually.\textsuperscript{54,55} It has been reported that Ag-NPs are more toxic than other metal NPs such as aluminum, iron, nickel, and manganese.\textsuperscript{56,57} Despite extensive research on their biological impact, there is currently a debate as to whether the silver ions or Ag-NPs or rather a combination of both contributes to their toxicity.\textsuperscript{58} We studied the effects of Ag-NPs in paper I-II.

**SPIONs**

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Figure 6. SPIONs functionalized with different coatings i.e. APTES, Chitosan, PEI and TSC (paper V)
Fe$_3$O$_4$-NPs are the main constituent of magnetic NPs, although other metals such as cobalt and nickel are also used. The unique superparamagnetic property of SPIONs makes them especially useful for medical applications. During alternating magnetic fields, the dipole moments of SPIONs have to be quickly reoriented and the energy loss can be dissipated as heat. This can consequently increase the temperature of the surrounding tissue, so called magnetic hyperthermia. Researchers are exploring using this effect for killing cancer cells$^{59}$. Furthermore, SPIONs are utilized in clinical trials as contrast agents in magnetic resonance imaging (MRI) as well as in the water treatment industry for removal of heavy metals and organic dyes$^{60-64}$.

Uncoated SPIONs are rarely used in industry. This is because they can agglomerate and precipitate under physiological conditions, which can lead to obstruction of blood vessels. Also Fe$_3$O$_4$, is not very stable and can readily undergo oxidation in the presence of air, light and moisture leading to unwanted cellular responses$^{65}$. Therefore in order to improve biocompatibility, biodistribution, and reduce their reactivity, SPIONs are normally coated$^{66,67,68}$.

Since the physicochemical properties of SPIONs are affected by the surface coating it is important to study the effects of surface coating on the toxicity of the SPIONs. We studied SPIONs functionalized with tri-sodium citrate (TSC), PEI, aminopropyl-triethoxysilane (APTES) and chitosan (Figure 6).

Chitosan is a biocompatible and biodegradable linear polysaccharide made from chitin. Due to its antimicrobial properties and affinity for many biomolecules, chitosan-SPIONs are used for biomedical applications$^{69}$. APTES is an important silane coupling agent that causes increase of protein and DNA adsorption on SPIONs$^{70}$. They can therefore be used for cell separation and drug targeting$^{71,72}$. TSC has anticoagulant properties due to its ability to chelate ionized calcium in blood$^{73,74}$. Finally, PEI is a polycation with high ionic charge density and large buffering capacity. It has been shown to disrupt endosomal osmolality leading to lysis and thereby release of PEI-SPIONS. PEI has high efficiency as a transfection reagent so PEI-SPIONs could be used for similar purposes$^{75,76}$.
3. Cardiovascular system deposition

NPs are a ubiquitous part of the environment and we are exposed to them in numerous ways, mainly through the respiratory tract, gastrointestinal tract (GIT) and possibly dermal absorption. Although NPs can cause diseases in primary exposed organs, a major concern is whether they could get into the circulatory system. The NPs can either cause cardiovascular disease or be subsequently distributed to secondary organs such as the liver and cause diseases there (Figure 7).

![Figure 7. Schematic presentation of possible bio-kinetics of NPs. GIT: gastrointestinal tract, CNS: central nervous system, BBB: blood brain barrier. The figure is inspired and modified from Oberdorster et al.](image)

3.1 Routes of entry to the cardiovascular system

Lungs
Airborne NPs are taken up in the lungs and respiratory tract. Upon inhalation, most of the NPs, depending on their physicochemical properties, deposit into the alveolar region where mucociliary clearance is impossible, and are thus taken up by macrophages. Small enough NPs (< 30 nm) are however
able to translocate rapidly from the lungs to lymph nodes and further distribute to the bloodstream and thereafter to other organs.\textsuperscript{79-81}

\textit{GIT}

On the other hand, NPs that can go through mucociliary clearance are swallowed and end up in the GIT.\textsuperscript{85} The ingestion of NPs present in food such as flavor enhancers and pigments also brings the NPs to this organ.\textsuperscript{82-84} The intestinal epithelial cells and the blood vessels are separated by several cell layers enabling the uptake of nutrient while hindering the uptake of macromolecules or NPs.\textsuperscript{77} Nevertheless, according to \textit{in vivo} studies, NPs can pass through the GIT into the blood and accumulate in the liver, spleen, and bone marrow.\textsuperscript{85, 86}

\textit{Skin}

Another possible route of entry to the bloodstream is through the skin. This largest organ of the body is composed of the epidermis, dermis and subcutis layers. The epidermis is composed of a 5-20 μm layer of constantly replaced dead keratinocytes over layers of living cells. The underlying dermis contains hair follicles, sebaceous glands and beneath that layer, the capillary vessels.\textsuperscript{77} There are generally two pathways for absorption of substances in the skin: along the skin appendages, or through the stratum corneum and the underlying layers. The appendages such as hair follicles are particularly important for uptake of NPs because of the depth they reach.\textsuperscript{87} In theory, only materials with appropriate octanol/water partition coefficient (representing polarity) and molecular mass (known as the “500 Dalton rule”) are able to penetrate intact skin. Therefore the uptake of normal insoluble NPs is unlikely and so far only passage of NPs though the upper layers of the epidermis has been reported.\textsuperscript{77, 89} The dermal absorption of USNPs as well as access to capillaries through wounds needs further investigation.

\textbf{3.2 NPs in the cardiovascular system}

\textit{NPs in the blood}

Upon entering the cardiovascular system the NPs are immediately in contact with and can thereby impact blood constituents. They can cause hemolysis of red blood cells, leading to anemia, jaundice and other pathological conditions. Also by activating the blood coagulation cascade, they can cause thrombogenicity.\textsuperscript{90} NPs in the blood can be coated with numerous proteins including immunoglobulins that can enhance recognition and uptake by cells of the reticuloendothelial system (RES).\textsuperscript{91, 92} This results in the entrapment of the NPs in organs of the RES with a high content of macrophages such as liver and spleen.\textsuperscript{93, 94}
**Endothelial cells**

NPs in the size < 20 nm can escape the RES absorption and possibly access the endothelium\(^{95}\). The highly versatile and multi-functional endothelial cells form a monolayer lining the entire vascular system. There they regulate many processes such as wound healing, blood flow and immune responses\(^{96}\). Therefore, any major disruption in the function or structure of the endothelium, can lead to cardiovascular diseases such as atherosclerosis and high blood pressure\(^{97}\). It has been reported that intravenous injection of mesoporous silica NPs to mice can cause fatality due to the obstruction in the vasculature\(^{98}\).

Important key players in cell structure and function are the lipids. Endothelial cells can synthesize and release vasoactive lipid mediators such as adrenic acid, isoprostanes and sphingosins\(^{99,100}\). In addition, endothelial cells respond to potentially harmful conditions by inducing coagulation and production of inflammatory mediators by the synthesis of ‘bioactive’ lipids such as eicosanoids\(^{101}\). It has been shown that NPs such as CuO-NPs are able to disrupt cellular lipid and cholesterol metabolism in hepatocytes\(^{102}\). The impact of NPs on endothelial lipid metabolism, however, and its consequences in physiology has not been fully investigated. Understanding the toxicity impact on endothelial lipids could elucidate the pathology and drive the development of new treatments of many cardiovascular diseases such as atherosclerosis\(^{103,104}\).

**Angiogenesis**

A vital function of endothelial cells is angiogenesis i.e. the formation of new blood vessels from a preexisting vascular network. It is the driving force of fetal organ development, wound healing and even involved in tumor growth and atherosclerosis\(^{105}\). Angiogenesis is stimulated by cytokines and growth factors that induce gene expression and proliferation of endothelial cells. This in turn stimulates the production of proteolytic enzymes that destroy the extracellular matrix, resulting in endothelial cell migration and invasion into tissues\(^{106}\). Other stimuli such as inflammation, ROS and hypoxia can also impact angiogenesis\(^{107,108}\). Therefore NPs that elicit these stimuli can have pro and anti-angiogenic properties. It has been revealed for example, that TiO\(_2\)-NPs can have anti-angiogenic effect with tolerable toxicity\(^{109}\).
Endothelial permeability

Endothelial cells are classified based on their intercellular junctions. Continuous endothelium is present in capillaries of the brain, skin and lungs. It is associated with a continuous basal membrane and has tight junctions (2 nm). The fenestrated endothelium has transcellular 50–60 nm pores sealed by a 5–6 nm diaphragm and is present in GIT and kidney glomeruli. Finally, discontinuous endothelium has a poorly structured basal membrane, contains 100-200 nm pores without a diaphragm and is found in liver, spleen and bone marrow.

The endothelium is thus a semi-permeable barrier between the blood and the surrounding tissues in charge of controlling the passage of solute and macromolecules. Small solutes move passively across the endothelium via paracellular route. Larger macromolecules on the other hand pass through either receptor or adsorptive transcellular pathways. (Figure 8)

In a state of acute or chronic inflammation (such as atherosclerosis, angiogenesis or tumor metastasis), mediators such as cytokines, growth factors and ROS can induce leakiness of endothelial layer and subsequent passage of plasma proteins. Under these circumstances, transport is also possible through endothelial vesicles/vacuoles. Therefore NPs that induce these inflammatory responses can impact the permeability of the endothelium, enabling their transport to other organs as well as disrupting endothelial functions. It has been shown, for example, that NPs can affect the permeability of the BBB and end up in the brain. Disruption of this vital tissue can lead to numerous neurovascular diseases.
3.1 Translocation of NPs

Figure 9. Mechanisms of NPs uptake in the liver. (A) Structure of liver sinusoid in hepatic lobules. (B) USNPs are able to extravasate different tissues nonspecifically. (C) Kupffer cells are able to take up larger sized particles or possibly even nanoparticle aggregates. (D) Fenestrated endothelium in the liver allows for uptake of larger particles. (E) Negatively charged NPs are optimally taken up by endothelial cells while (F) NPs with positive surface charge tend to be taken up by hepatocytes. Image with permission from Wang et al.115.

As discussed, the inherent or induced permeability of the endothelium could enable the translocation of NPs. One of the major organs where NPs can accumulate during transfer through the bloodstream is the liver. Studies on this matter have demonstrated high accumulation and retention of NPs in liver after injection or digestion causing hepatic injuries and diseases.116-118 NPs that enter the sinusoids (the terminal vessels between hepatocyte) are taken up by specialized macrophages (Kupffer cells) and accumulate in the liver.115 (Figure 9) Hepatocytes constitute roughly 80% of the mass of the liver and perform many metabolic, endocrine and secretory functions. These cells are exceptionally active in the synthesis of protein and lipids for export. In vivo studies have reported that ZnO-NPs and Ag-NPs exposure could lead to their accumulation in the liver where they can cause toxicity, albeit at high concentrations.119-121 The transfer of NPs to other body fluids such as breast milk and amniotic fluid has also been demonstrated. Au-USNPs and to a lesser extent NPs (18 nm) are evidently able to translocate from maternal blood into the fetus in rats.70 However, not all NPs stay in the circulatory system or translocate to organs, NPs can be cleared from the body through renal excretion.122

Finally, it should be noted that the biodistribution of NPs is strongly dependent on their size and other physicochemical properties.
4. Physicochemical properties govern biological impact

The cellular exposure to NPs can cause a plethora of stress responses, including cytotoxicity, genotoxicity, oxidative stress, protein aggregation, and disruption of lipid metabolism\textsuperscript{123-125}. The physicochemical properties of NPs determine their biological effect at both the cellular and organismal level\textsuperscript{126-128}. Here some of those most prominent features of the NPs are presented. However, it should be emphasized that none of these properties alone decide the outcome of NPs exposure. Combinations of the physicochemical properties of the NPs as well as their exposure environment ultimately govern their biological impact.

Figure 10. (A) Physicochemical properties of NPs impact their effect on the cells and organism; (B) Image adapted from Auffan et al\textsuperscript{94}
4.1 Size and curvature

The size of the NPs can affect their biodistribution, cellular uptake and localization. With regards to the first concern, evidently at 20 nm Ag-NPs localize mainly in the liver, whereas at larger sizes they accumulate in the spleen\textsuperscript{129,130,93}. USNPs can extravasate different tissues nonspecifically since the intercellular space of gap junctions is about 4 nm (Fig. 6B)\textsuperscript{131}. An \textit{in vivo} study has demonstrated that TiO$_2$-USNPs, in the size range of the tight junctions, can possibly pass through BBB\textsuperscript{35}.

Regarding the cellular uptake of the NPs, their internalization is also affected by other properties such as membrane-NPs binding energy, protein or ligand density on the surface of NPs, and nanoparticle curvature\textsuperscript{132}. The interaction of NPs with the cell membrane and subsequent uptake can be receptor-ligand mediated or via unspecific interactions\textsuperscript{133,134}. The routes of uptake include phagocytosis, micropinocytosis, endocytosis or even diffusion\textsuperscript{135}. NPs at 50-60 nm could recruit sufficient number of receptors to successfully trigger internalization through receptor-ligand mediated endocytosis\textsuperscript{136}. The route of active uptake is different depending on the size of NPs. The uptake of 45 nm Au-NPs reportedly involve clathrin-mediated endocytosis, while at the size of 13 nm phagocytosis was mostly observed\textsuperscript{137}. In case of USNPs in addition to membrane diffusion, they can also accumulate on the plasma membrane before gradually entering the intracellular region\textsuperscript{138}.

Finally, the cellular localization of the NPs is affected by their size. In general, after the uptake of NPs in the early endosomes, NPs move along microtubules toward the cellular interior, fuse with late endosomes and finally lysosomes where they are stored. Under specific conditions, such as cell stress, the lysosomes can undergo exocytosis releasing their undigested content\textsuperscript{139}. Depending on their size NPs, and especially USNPs, can escape the vesicular system to the cytosol\textsuperscript{140}. In this case USNPs are able to enter the nucleus through nuclear pores (2.5 nm)\textsuperscript{141,142}. Once inside the nucleus, the USNPs can interact with DNA and cause DNA strand breakage. Larger NPs can also enter the nucleus during cell division\textsuperscript{143,141}.

Another important property of the NPs is their curvature. It has been shown that the apparent pKa of bound ligands to Au-NPs increases with increasing NP curvature/size (4-7 nm). The rationale for this effect was described as follows: larger NPs have less curvature, subsequently the average distance between the head-groups of deprotonated ligands decreases. This leads to stronger electrostatic repulsions between the ligands, which is energetically unfavorable. The system will thus respond by “regulating” the ligands charges by shifting the acid–base equilibrium toward the protonated state. Consequently, the fraction of charged ligands decreases and the apparent pKa increases\textsuperscript{144}. According to this study rationalizing the spatial distributions of H$^+$, OH$^-$ or counter ions is possible. They predict that the pH be-
ing one to two units lower, closer to the NP surface than in the solution’s bulk. Geometric curvature of the NPs also controls the chemical patchiness and self-assembly of NPs.\textsuperscript{145}

So far only the core size of the NPs has been discussed, but NPs in suspension can join into larger structures. These are termed agglomerates, (held together by weak forces such as van der Waals), or aggregates if they are bound together by stronger forces, such as covalent or metallic bonds.\textsuperscript{146} Studies have shown that the aggregation or agglomeration state of the NPs could influence their biological impact due to the variations in size, and reduction of the total surface area of the NPs.\textsuperscript{11,147}

The size of the NPs also affects their interaction with proteins as it will be discussed later.

4.2 Shape and crystal structure

In addition to size, the shape of the NPs also affects their cellular uptake and localization. For example the preferential cellular uptake of hydrogel nanodiscs over nanorods has been reported.\textsuperscript{148} A recent study demonstrated that the morphology of NPs, regardless of their surface chemistry, size, or composition determine their cellular fate. They found that NPs with sharp shapes could pierce the membranes of endosomes and escape to the cytoplasm.\textsuperscript{140} The crystal structure of NPs can affect their oxidative activity and subsequent toxicity, as discussed earlier for anatase and rutile TiO\textsubscript{2}-NPs.\textsuperscript{30}

4.3 Surface charge

Positively charged NPs cause higher disruption of the plasma membrane integrity, which thereby enhances their cellular uptake and cause a stronger cytotoxic response compared to neutral and negatively charged NPs.\textsuperscript{149} Neutral or negatively charged NPs on the other hand have been shown to have prolonged blood circulation time.\textsuperscript{150} Lockman et al. observed that the surface charge of NPs can alter the BBB integrity and permeability with positive charged NPs causing immediate toxicity.\textsuperscript{151} Moreover, highly positively charged polyplexes can associate with negatively charged cell surface heparan sulfate proteoglycans inducing phagocytic like mechanisms for internalization.\textsuperscript{152} In the following section the effect of surface charge on the protein corona will be discussed in more detail.

4.4 The protein corona

When NPs enter biological fluids they are immediately covered by the biological components present. This protein corona may impact the physicochemical properties of the NPs by inducing phase transformations and free
energy releases\textsuperscript{133}. Subsequently the interaction of NPs with the biological system is altered\textsuperscript{153}.

**Structure of the protein corona**

The structure of the protein corona is dynamic. Loosely bound proteins will form the initial soft corona driven by protein-protein interactions. With time, a denser corona of higher affinity proteins will be formed (hard corona), which is strongly attached to the NP\textsuperscript{154-156}. One hypothesis is that the hard corona proteins interact directly with the NP surface while the soft corona proteins interact with the hard corona via weak protein–protein interactions\textsuperscript{157}.

According to Vroman’s theory, the dynamic adsorption pattern of blood proteins to foreign inorganic surfaces is as follows: abundant proteins such as albumin and fibrinogen may initially occupy the surface and get subsequently replaced by other proteins with higher binding affinity for the surface\textsuperscript{158}. However the Vroman theory is not always applicable to NPs. While the theory was confirmed for SWCNTs (fibrinogen followed by immunoglobulin, transferrin, and albumin), SPIONs <50 nm did not follow the pattern when exposed to plasma proteins\textsuperscript{159, 160}. These results emphasize the complicated interaction between NPs and proteins which is affected by the intrinsic properties of NPs, the ratio between NPs and proteins, temperature and the composition of the suspension\textsuperscript{155, 161}.

It has been observed that even at low plasma concentrations, there is a complete surface coverage by the corona layer\textsuperscript{162}. This could be potentially problematic for NPs with surface coatings that are used in medical applications. However, it has been shown that the adsorbed corona does not completely mask the surface of NPs or its functional groups. Studies have reported that the surface of dextran-coated SPIONS could be recognized by macrophages, despite the plasma corona formation, and that cellular uptake was ultimately determined by the nature of the surface coatings\textsuperscript{163, 164}.

**Physicochemical properties of NPs impact protein corona**

In general curved NPs surfaces provide enhanced surface area to the adsorbed protein molecules\textsuperscript{165}. The size of the NPs compared to the proteins may impact the stability of the protein corona. For NPs and proteins of similar size, the protein corona formation may be reversible and unstable compared to larger NPs\textsuperscript{166}. For example, negatively charged polyacrylic acid Au-NPs at 12 nm can bind to fibrinogen with higher affinity compared to 7 nm NPs\textsuperscript{167}.

The surface charge of the NPs also impacts protein absorption. In cell culture medium, it was shown that 30-36 % of the negatively charged groups of the protein bovine serum albumin (BSA) form bonds with the protonated and charged aluminium oxide NPs surface. Thereafter, additional BSA binds onto this monolayer as dimers\textsuperscript{168}. NPs with no charge bind less protein
than their negatively charged (COOH functionalized) or positively charged (NH$_2$ functioned) counterparts. Positively charged NPs have been shown to preferentially adsorb proteins with an isoelectric point (pI) <5.5 such as albumin, while the negative surface charge bind those with pI > 5.5 such as IgG. NP surface can thus be modified to avoid adsorption of proteins using polyethylene glycol (PEG). This so called PEGylation of NPs imparts a “stealth character” to an NP surface, thus shielding it from being recognized by immune cells and increases its blood circulation time.

**Modification of proteins**

The surface of NPs can also modify the structure and thereby function of the adsorbed protein, thus affecting the overall bio-reactivity of the NPs. These modifications of the secondary structure of proteins are affected by the curved surface of NPs as well as the properties and structure of individual proteins. In this regard, the hard and soft protein corona also represents proteins that resist adsorption or conformational changes, and that readily undergo conformational changes respectively. Studies have shown that TiO$_2$-NPs can cause conformational changes and reduce polymerization of the cytoskeletal protein tubulin. In case of ZnO-NPs however, only minor conformational changes to BSA were reported. Interestingly, SPI-ONs have been shown to induce irreversible conformational changes to transferrin, an important carrier of iron.

These conformational changes may affect downstream protein-protein interactions, cellular signaling and DNA transcription. The NPs may cause enzymes to lose their catalytic activity, but they may also increase the accessibility of the active site for its substrate. SWCNTs exposure to α-chymotrypsin for example, resulted in loss of enzymatic activity. On the other hand, covalently bound horseradish peroxidase and lysozyme on SWCNTs was able to retain their native structure and activity even under denaturing conditions.

Moreover, the NPs may induce abnormal unfolding of the bound proteins to form novel conformational epitopes or induce the exposure of hidden epitopes resulting in elicitation of an unwanted immune response. Finally, these structural changes may also lead to loss of tolerance against self thereby provoking autoimmune responses. Once again the size of NPs plays an important role. It has been shown that larger silica NPs (100 and 15nm) induced a higher change in the secondary structure of proteins compared to smaller 4 and 6 nm NPs.

One suggested mechanism behind protein aggregation at NPs surface is that NPs can enhance the appearance of a critical nucleus for nucleation of protein fibrils, as it has been shown for SWCNTs interacting with human beta(2)-microglobulin.
An important consequence of the possible NPs induced protein misfolding is ER stress. Accumulation of these misfolded proteins in the ER triggers the unfolded protein response (UPR). This event induces the production of chaperones to facilitate protein folding, enhanced proteosomal degradation of misfolded proteins and eventually apoptosis. In addition, UPR activation plays a critical role in lipid metabolism and homeostasis leading to diseases such as dyslipidemia and cardiovascular disease.

4.5 Dissolution

The dissolution rate depends on the solvent properties (such as pH and ionic strength) and on the properties of NPs (such as aggregation states). The dissolution kinetics are also proportional to the specific surface area, with smaller NPs exhibiting faster dissolution than larger particles. The protein corona may also influence the dissolution rate.

Internalized soluble NPs can be degraded into ions by the hydrolyzing enzymes in the lysosomes at low pH. This Trojan horse like effect delivers ions inside the cells. In general the dissolution of NPs can lead to i) protein dysfunction (e.g. destruction of Fe-S cluster, exchange of catalytic/structural metal), ii) production of ROS (Fenton reaction), iii) impair membrane function and finally iv) genotoxicity.

In this regard, essential trace elements such as iron, Zn and Cu are especially important as they can influence gene expression. Therefore soluble CuO-NPs, ZnO-NPs and SPIONs can cause disruption of cellular homeostasis. The mechanism behind their action is discussed here in more detail.

Cu ions

Generally, upon exposure to an excess of Cu ions from CuO-NPs two events occur rapidly in hepatocytes: i) internalization of Cu transporter 1 (hCtr1) at the plasma membrane through vesicles, thereby stopping Cu entry; ii) recruitment of a Cu-ATPase (ATP7B) in the trans-Golgi network membrane to transfer Cu into the bile. The excess Cu can potentially concentrate in the cytosol and the nucleus. High Cu levels in the nucleus block the activity of nuclear receptors (through binding or oxidation) leading to down regulation of transcripts involved in cholesterol metabolism, such as HMG-CoA reductase (HMGCR) and HMG-CoA synthase (HMGCS1). This subsequently leads to low total cholesterol in the liver. In hepatocytes, Cu selectively up-regulates molecular machinery associated with the cell cycle and chromatin structure and down-regulates cholesterol biosynthesis. In some cases, an increase of cellular lipid droplets (LDs) has been observed, although it was shown to be transient due to functioning ATP7B. Exposure to Cu ions released from CuO-NPs or inactivation of ATP7B (for example, in patients with Wilson’s disease) can lead to
dysregulation of cellular lipid metabolism (Figure 11). In addition, Cu ions can impact the mitochondria releasing ROS\textsuperscript{193}.

![Figure 11](image.png)

Figure 11. The effect of CuO-NPs on cells. The Cu ions released from CuO-NPs are taken up by Ctr1 and can be removed by ATP7B. However, upon uptake in cells and subsequent lysosomal degradation, excess Cu ions in the cytosol and nucleus can overwhelm the homeostasis response leading to a decrease of enzymes in cholesterol metabolism or disruption of mitochondrial membrane integrity leading to release of ROS\textsuperscript{191}.

**Ag ions**

Ag ions are known to inhibit, thiol group-containing enzymes, such as NADH dehydrogenase II in the respiratory system, by binding to thiol groups leading to the denaturation of the enzyme. Moreover, they can prevent replication of DNA by its condensation.

**Zn ions**

Generally, in excess of Zn, increased expression of genes required for zinc efflux, storage, and in eukaryotes compartmentalization occurs. In *S. cerevisiae* vacuoles are major site of zinc storage and the stored Zn can be utilized by as many as eight generations of progeny cells under Zn starvation\textsuperscript{196}. It has been shown that free Zn ions are not a major contributor for ROS generation\textsuperscript{197}.
4.6 ROS production

The ions released can also cause the production of ROS, which could lead to oxidative stress. The Cu as well as free iron ions (Fe$^{2+}$) from SPIONs can react with hydrogen peroxide producing hydroxyl radicals and ferric ions (Fe$^{3+}$) via the Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^-.$$ 

Therefore NPs can cause oxidative stress through their intrinsic oxidative activity or by disruption of mitochondria$^{127}$. Although under normal physiological conditions the generation of low amounts of ROS in mitochondria is normal and neutralized by antioxidants, but an excess of ROS can overwhelm the defense system, leading to oxidative stress and induction of inflammatory and pro-apoptotic factors$^{127,198}$. In general, the mechanism of oxidative stress induced by NPs is as follows: during conditions of mild oxidative stress, transcriptional activation of antioxidant enzymes occurs. At an intermediate level, redox-sensitive mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) cascade a pro-inflammatory response. High levels of ROS result in mitochondrial membrane damage affecting the electron transport chain, induction of cytochrome P450 enzymes, as well as DNA damage$^{201,143}$. Moreover, membrane damage through lipid peroxidation is possible$^{202,174}$. 


5. Methodology

Detailed information regarding each assay is described in the paper I-V. In general, the methods have been used to: 1) characterize of the physico-chemical properties of the NPs, 2) assess their \textit{in vitro} cyto- and genotoxicity and ultra-structural impact 3) study the effect of NPs on angiogenesis, 4) study the NPs impact holistically, and finally 5) assess \textit{in vivo} toxicity. In this section, a brief description of the desired endpoint and some of the methods used is provided.

5.1 Characterization of NPs

Overview of the endpoints and methods used for the physicochemical characterizations of NPs (Paper I-V) is shown in figure 12. For NPs in suspension, the measurements were performed on NPs suspended in sterile de-ionized water and in the cell culture media used in each paper. Also during the measurements, the temperature was adjusted to what was used for the \textit{in vitro} and \textit{in vivo} studies in order to mimic the exposure conditions. Before each experiment the NPs were suspended in appropriate media and sonicated using a sonicator bath for 30 minutes at 40 kHz and vortexed vigorously to avoid aggregation.

![Figure 12. The endpoints and methods used for the characterization of NPs in both powder form and in suspension.](image-url)
Hydrodynamic size

The hydrodynamic size is measured using dynamic light scattering (DLS) and is representative of the size of hydrated/solvated NPs\textsuperscript{203}. DLS employs Stokes-Einstein equation where the time dependent fluctuations in the scattering intensity from NPs in Brownian motion are measured. This provides the diffusion coefficient (DT) and thereby the hydrodynamic size. The experiments were performed on a Malvern Zetasizer Nano series instrument with a He-Ne laser at 633 nm. Stokes-Einstein equation:

\[
D_h = \frac{k_B T}{3\pi \eta D_t}
\]

- $D_h$ = hydrodynamic size
- $D_t$ = translational diffusion coefficient
- $k_B$ = Boltzmann’s constant
- $\eta$ = viscosity
- $T$ = temperature

Zeta potential

The surface charge of the NPs is measured by zeta potential ($\zeta$P). The charge on the surface of NPs attracts a thin layer of opposite charged ions (stern layer). This ionic double layer moves with the NPs as it diffuses through the medium. The electrical potential at the periphery of the double layer (i.e. the slipping plane) is known as zeta potential and is representative of the surface charge as well as colloidal stability. Typically the zeta potential value has a range of +/-100 mV to -100 mV. NPs suspensions with a value higher than +/-30 mV are characterized as having good colloidal stability\textsuperscript{204,205}. The Henry equation and the measurement of the electrophoretic mobility of the NPs, is used for the calculation. Similar to DLS measurement, a Malvern Zetasizer Nano instrument was used with a He-Ne laser at 633 nm.

Figure 13. Schematic presentation of the electrical double layer, zeta potential, and Henry equation. Image adapted from Malvern.
Surface area
The surface area of TiO$_2$-NPs in paper III was measured using the particles Brauner-Emmett-Teller (BET) method using a micromeritics volumetric adsorption analyzer. The BET measurements of specific surface area are based on the adsorption of nitrogen atoms on the surface of NPs. Since the measurement was performed in powder form, the aggregation and/or agglomeration of the NPs would not affect the surface area determination$^{206}$.

Intrinsic oxidative ability
To study the intrinsic oxidative potential of the NPs, a cell free method with some modification was utilized$^{207}$. The compound, 2’, 7’ dichlorofluorescein diacetate (DCFH-DA) is normally used as a marker of oxidative stress and it can also be used to assess the oxidative activity of the NPs. In a cell system, DCFH-DA is hydrolyzed by intracellular esterases thus producing dichlorofluorescein (DCF) which in turn can be oxidized to fluorescent 2’, 7’ dichlorofluorescein (DCF). This hydrolysis can be chemically performed by NaOH, followed by incubation with NPs at appropriate temperature and concentration for a cell-free assessment. The DCFH oxidation can then be measured at 485-530 nm (Paper I-V).

Ion leakage assessment
Inductively coupled plasma- mass spectrometry (ICP-MS) was performed to assess the amount of ions released from NPs in the medium. Briefly, the samples were ultra-centrifuged at 100000 g for 1 h. ICP-MS was performed on the collected supernatant and none-centrifuged NPs, which were diluted (10 and 500 times respectively) to be used for comparison before measurement with ICP-MS. The internal standard used was rhodium at 10 ppb.

5.2 Biological test systems

Saccharomyces cerevisiae
This unicellular eukaryote organism provides a low-cost, high-throughput in vitro system for toxicity assessment. Two strains were used, wild type GenC01, and GenT01, where the promoter for a DNA repair gene (RAD54) is copied and placed in front of a green fluorescent protein (GFP) gene, thus producing fluorescence upon chromosomal damage (paper I)$^{208}$.

Primary mouse hepatocytes
To assess the hepatic toxicity of NPs, primary hepatocytes from C57/6J mice were used. Primary cells provide cultures that have characteristics very close to the tissue of origin. The cells were isolated by a collagenase and perfusion technique and cultured on fibronectin-coated plates as described
by Palacios et al.\textsuperscript{209,210}. Animal procedures were approved by the University of the Basque Country and Animal Care and Use Committees. Description of the cell culture media is provided in paper II.

**HMEC-1 cells**

Endothelial cells were chosen in order to study the effects of NPs on the endothelium (Paper III and IV) as well as their effect on angiogenesis (Paper III). HMEC-1 cell lines were established by Ades et al., and obtained from human dermal microvascular endothelial cells\textsuperscript{211}.

**Zebrafish embryos (Danio rerio)**

In paper III, transgenic Tg (fli1a:EGFP)y1 zebrafish embryos, in which endothelial cells are labeled with EGFP\textsuperscript{212} were used as an in vivo model organism. The embryos were maintained according to standard protocols at zebrafish facilities at Karolinska Institutet or Linköping University. Natural mating overnight in breeding aquaria produced embryos, and eggs were collected immediately after spawning and kept at 28.5°C in humidified ambient air in incubators. In order to emulate environmental exposure to NPs in aquatic systems, the embryos were exposed to NPs in water at 1- cell stage at 0 hour post fertilization (hpf) or 48 hpf. Additionally, in order to observe the effects of internalized NPs on embryo development and angiogenesis, the embryos were injected either at 0 hpf, or in circulation at 48 hpf. At 24 hpf, the major vessels, aorta (dorsal artery), and posterior cardiac vein are formed through vasculogenesis and the vascular architecture is largely established at 72 hpf\textsuperscript{213}. Toxicity was evaluated as gross changes in development and defective morphogenesis. Dead embryos were characterized as by “rotten” black spots in the chorion alternatively grey and still embryos if exposure started at 48 hpf. All analysis was done at 5 dpf (120 hpf).

### 5.3 Toxicity assessment

**Cytotoxicity assessment**

Different cytotoxicity assays were used in the papers, depending on the type of cells and NPs and desired endpoints. The cytotoxicity induced by NPs was assessed based on mitochondrial activity (MTT assay, paper II and V), membrane integrity (LDH assay, paper III), mitochondrial membrane potential (TMRE, paper V), and nuclear morphology (Hoechst, paper V).

**Genotoxicity**

In order to assess the DNA damage the Comet assay (single-cell gel electrophoresis)\textsuperscript{214} was used. In brief, cells are lysed with detergent resulting in the formation of nucleoids containing supercoiled loops of DNA linked to the nuclear matrix. Electrophoresis in alkaline buffer on the cells, results in comet-like structures, because strand breakage causes DNA to lose the
supercoiling and become free to extend toward the anode. The comets after fluorescent staining (SYBR Green) can be visualized by fluorescence microscopy. The intensity of the comet tail relative to the head reflects the number of DNA breaks. The DNA damage is reported as the Olive tail moment (OTM)\textsuperscript{215}.

\[ \text{OTM} = (\text{Tail mean} - \text{Head mean}) \times \% \text{DNA in tail} \]

**Intracellular ROS/Nitric oxide (NO)**

As with the cell free ROS system DCFH-DA was used to assess the intracellular ROS as well as a Rhodamine probe for superoxide detection provided by manufacturing company. (Excitation/Emission= 550/610 nm) (Paper III and V).

Measurement of NO on the other hand, is difficult due to its volatile nature. Therefore by measuring two stable breakdown products, nitrate (NO\textsubscript{3}) and nitrite (NO\textsubscript{2}) by photometric means, the amount of NO in the cells can be measured indirectly (paper III).

**Uptake and ultrastructural impact**

*Transmission electron microscopy (TEM)*

TEM was used to study the interaction of NPs with cells, by visualizing their uptake, localization and the impact on the cellular ultrastructure. (Papers I, III and V) TEM imaging was prepared according to standard protocol.

*Hyperspectral imaging*

The uptake and localization of NPs in the zebrafish embryos were assessed using ultrahigh resolution imaging employing the darkfield CytoViva condenser. This particular illuminator focuses fixed-geometry, highly collimated light at oblique angles on samples thereby dramatically improving contrast and signal-to-noise ratio. The system allows for optimized resolving power and is thus capable of detection of non-fluorescing samples including non-labeled NPs. The hyperspectra of zebrafish embryos exposed to NPs were filtered against control samples. Pixels whose spectra deviated from the control spectral library were hence identified as NPs (Paper III).
5.4 Angiogenesis assessment

Figure 14. (A) Angiogenesis and formation of lumen adapted from Kamei et al. 216 (B) In vitro tube formation assay protocol.

In order to study the effect of NPs on angiogenesis in paper III both in vitro (3D cell culture of endothelial cells) and in vivo model (zebrafish embryos) were used. For the in vitro study, a tube formation assay was employed (Figure 14B). When cultured on a basement membrane extract (BME) endothelial cells differentiate and form tube-like structures possibly containing a lumen formed from fusion of pinocytic vesicles217 (Figure 14 A). Therefore with this method the uptake of the NPs inside the vesicles and subsequent location in the lumen as well as effects on tube formation can be assessed.
5.5 Omics analysis

In this thesis, three omics approaches were utilized namely proteomics and lipidomics on mouse hepatocytes (paper II) and transcriptomic using next generation sequencing on endothelial cells, and qPCR targeting specific genes on zebrafish embryos using qPCR (paper III).

Proteomics

Figure 15. Schematic overview of 2D-DIGE

In paper II, after the exposure of the hepatocytes to NPs, proteins were extracted and analyzed with 2D-difference gel electrophoresis (DIGE), quantified by an imaging software and the differentially expressed proteins identified by nLC-ESI-MS/MS (Figure 15). DIGE allows the evaluation of the differences in proteomic profile between cells in different states or exposure conditions.

MS analysis for protein identification was performed on nano-LC-MS/MS after protein spot excision and trypsin in-gel digestion. Columns packed with reverse-phase C18 were used for separation. The proteins were identified in Swissprot using Mascot Server.

Lipidomics

Thin layer chromatography (TLC) was used for the analysis of the lipids of hepatocytes in paper II exposed to NPs. In principle, lipids with different solubility and strength of adsorption to the adsorbent will travel up the plate in different extent. Then the separated components of the mixture can then be visualized\textsuperscript{218}. Standard curves for all lipid classes can be prepared and lipid spots can be quantified using software\textsuperscript{219}.
Transcriptomics

Next generation sequencing (NGS), RNA-seq

In order to study the effects of NPs on the transcriptome of endothelial cells (paper IV), NGS (RNA-seq) was used which applies high-throughput sequencing of complementary DNA (cDNA) delivering a transcriptional profile of the cells. NGS facilitates the identification of structure of genes, their splicing patterns, post-transcriptional modifications as well as mutations. Moreover mRNA abundance can explain ca 40% of the variation in the levels of protein accumulation. Another advantage of NGS compared to classical microarrays is that it could be used for the discovery of genes and expression profiling of organisms without reference genome by de novo assembly of short reads generated.

Determination of target gene expression by (RT-qPCR)

In paper III, the differential expression of some target genes in zebrafish embryos injected with TiO$_2$-USNPs and non-exposed was assessed using RT-qPCR. Total RNA expression for target RNAs vascular endothelial growth factor Aa and c (VegfAa, Vegfc), Interleukin-1 beta (Il1β), Tumor necrosis factor alpha (Tnfa), and p53 was compared to a constitutively expressed gene (i.e., β-actin) in the mRNA samples from untreated and treated embryos.

Bioinformatics analysis

The results obtained from the NGS analysis was further analyzed by determining gene ontology (GO) categories i.e. biological process, molecular function and cellular compartment. The GO enrichment analysis was performed using Reactome. The GO categories with p<0.01 and at least three significant genes are reported.
5.6 Integrated nanoimpact index (INI)

In paper V, INI was used to compare the toxicity of SPIONS with different coatings using different toxicology endpoints such as cytotoxicity, genotoxicity as well as intracellular and intrinsic ROS production. Two different cell types i.e. keratinocytes and endothelial cells were used representing primary and secondary routes of exposure respectively. INI is based on integrated biomarker response (IBR)\textsuperscript{224}. Briefly, the INI was calculated by

1. Calculation of mean (X) and standard deviation (SD) for each SPIONs response in each assay
2. Calculation of mean value for all SPIONs responses in each assay (M)
3. Standardization of data for each assay calculated as
   
   \[ Y = \frac{(X - M)}{SD} \]

4. \( Z = Y \) (stimulation) or \( Z = -Y \) (inhibition), based on biological effects
5. The minimum value for all SPIONs for each assay was added to Z calculating score S and \( l_{\text{min}} \) as the absolute value
   
   \[ S = Z + l_{\text{min}} \]

6. The values obtained were visualized using star plot in which the radial coordinate corresponds to the values
7. The INI value was calculated as the area of each star plot:
   
   \[ A_i = \frac{\left(S_i \times S(i + 1)\right) \times \sin\left(\frac{2\pi}{n}\right)}{2} \]

Where \( n \) is the number of assays in each plot.

\[ \text{INI} = \sum_{i=1}^{n} A_i \]
6. Summary of papers

In general, my PhD work can be divided into three sections: 1) assessing the toxicity and biological impacts of metal and metal oxide NPs on yeast cells, and then more in detail on hepatocytes (paper I and II), 2) a more focused study on the vascular toxicity and effects of TiO$_2$-USNPs, TiO$_2$-NPs and SWCNTs (paper III and IV), and finally 3) assessing the toxicity of functionalized SPIONs in order to develop INI (paper V).

Paper I. The effects of engineered nanoparticles on the cellular structure and growth of Saccharomyces cerevisiae

In this study, a screening of the toxicity of some of the most widely used NPs in industry i.e. CuO-NPs (< 50 nm), ZnO-NPs (< 100 nm), Ag-NPs (<10 nm), SWCNTs as well as TiO$_2$-USNPs (1-3 nm) were performed using S. cerevisiae. Initially, in order to understand which physicochemical properties govern biological impact, the NPs were thoroughly characterized to assess their intrinsic oxidative ability (cell-free ROS assay), release of ions (ICP-MS), hydrodynamic size (DLS) and surface charge (zeta-potential). Then the cytotoxicity and genotoxicity of the cells were evaluated. The findings were compared to that of their respective bulk forms in order to understand whether the nanometer size or particle composition governed the results. Finally, we studied the effects of NPs on the cellular ultrastructure by TEM. We found that in general the NPs were more toxic and reactive than their bulk form.

The characterization results showed that among the NPs studied, CuO-NPs were the most reactive producing both ROS and releasing ions. In agreement with their reactivity, they exhibited the highest cytotoxicity by reducing the cell density almost 80 %. Based on TEM images, they induced the increase of LD formation, possibly by overwhelming the Cu ion homeostasis defense mechanism. CuO-bulk also had oxidative activity and was potentially toxic at higher concentrations.

ZnO-NPs released ions, but the oxidative activity was only observed in the bulk form. Despite lack of cytotoxicity, there was a considerable increase
in the size of vacuoles, indicating the defense mechanism of yeast cells to store excess Zn ions.

Ag-NPs did not release ions and information about their oxidative activity at higher concentrations could not be assessed. Nevertheless, these NPs decreased cell viability by almost 40 %. According to TEM images, Ag-NPs disrupted intracellular components and their uptake and intracellular localization inside vesicles could also be clearly observed.

Interestingly TiO$_2$-USNP surface charge changed from positive to negative in water and media respectively; also they did not produce ROS nor released ions. Their cytotoxicity was only about 35 %. The TEM images showed an increase of intracellular dark deposits throughout the cell which could be internalized TiO$_2$-USNPs. In addition, the impact on nuclear morphology could be observed, indicating early cytotoxic response. TiO$_2$-bulk was not cytotoxic.

Among the NPs studied, SWCNTs had the highest oxidative activity. They did not, however, cause significant cytotoxicity. The TEM images showed no uptake or ultra-structural impact of the SWCNTs, which suggests that due to lack of a subsequent Trojan horse effect, the intrinsic ROS production by the SWCNTs were not strong enough to elicit toxicity.

In summary, we could show the reactivity of NPs compared to their bulk form as well as the effects of the physicochemical properties of the NPs on their biological impact.

Paper II. Proteomic and lipidomic analysis of primary mouse hepatocytes exposed to metal and metal oxide nanoparticles

Primary hepatocytes isolated from mice were treated with low doses (1 and 5 mg/l) of TiO$_2$-USNPs as well as ZnO-NPs, CuO-NPs and Ag-NPs for 48 h. The NPs were characterized in hepatocytes cell culture media. The cytotoxicity of the NPs was studied using MTT assay. The quantitative proteomics analysis was done using 2D-DIGE and nLC-ESI-MS/MS, and lipidomic analysis was performed by TLC.

The cell-free ROS assay agreed with findings in paper I, with the exception of ZnO-NPs that exhibited significant oxidative activity in hepatocytes cell culture media. It is, however, not clear whether they also induce intracellular ROS production. At these concentrations only CuO-NPs and Ag-NPs exhibited cytotoxicity at 5 mg/l.

The identified proteins were mostly from the mitochondria. The ranking of the effects of NPs on protein expression was CuO-NPs > Ag-NPs > ZnO-NPs > TiO$_2$-USNPs in agreement with their toxicity. The none-cytotoxic NPs
(TiO$_2$-USNPs and ZnO-NPs) showed an almost concentration independent impact on the proteins while logically, more proteins were differentially expressed for CuO-NPs and Ag-NPs at the cytotoxic concentration.

In general, the proteins identified were involved in the urea cycle, lipid metabolism, electron transport chain, glucose metabolism, signaling, cellular structure as well as some nuclear proteins. Most of the proteins were affected by several NPs, but only ZnO-NPs and TiO$_2$-USNPs affected unique proteins. TiO$_2$-USNPs caused the over expression of ATP-synthase and electron transferring protein alpha (ETF$\alpha$) indicating a cell in need of energy, while ZnO-NPs exposure caused the down expression of RNA helicase.

Lipidomics analysis showed that CuO-NPs had the highest impact on lipids and decreased phosphatidylethanolamine and phosphatidylinositol as well as possibly causing membrane integrity disruption. Ag-NPs exposure increased total cellular lipids and triacylglycerol (TG) and decrease of sphingomyelin (SM). TiO$_2$-USNPs also caused a decrease of SM. ZnO-NPs exposure did not affect the cellular lipids.

**Paper III. Vascular toxicity of ultra-small TiO$_2$ nanoparticles and single walled carbon nanotubes *in vitro* and *in vivo***

The aim of this study was to study the effect of TiO$_2$-USNPs on endothelial cells *in vitro*, and zebrafish embryos *in vivo*. The findings were compared to 30 nm TiO$_2$-NPs as well as SWCNTs.

The characterization of TiO$_2$-USNPs and SWCNTs in endothelial cell culture agreed with previous studies. TiO$_2$-NPs did not have significant oxidative activity and did not induce intracellular ROS production. However, some intrinsic ROS production in cell culture media was observed. Their surface area was 50 m$^2$/g compared to 470 m$^2$/g for TiO$_2$-USNPs. Only SWCNTs caused intracellular ROS production.

TiO$_2$-USNPs were not cytotoxic, but were genotoxic *in vitro*. TiO$_2$-NPs exhibited some cytotoxicity at high concentrations (around 10 %) and were also genotoxic *in vitro*. SWCNTs were the most cytotoxic but only 20 % at 40 mg/l *in vitro* but were not genotoxic.

Only TiO$_2$-USPs showed anti-angiogenic effect both *in vitro* and *in vivo*. TiO$_2$-NPs and SWCNTs did not impact angiogenesis even though their uptake inside the tubes was visible. As shown in figure 15 in methodology, due to their uptake inside vesicles, the NPs can end up in the lumen of angiogenic tubes. Indeed, cellular uptake and localization inside vesicle was shown in TEM images. In addition, the presence of free TiO$_2$-USNPs in the cytosol could also be seen.
Only TiO$_2$-USNPs induced severe adverse effects in vivo. Zebrafish embryos exposed to 1000 mg/l (470 m$^2$/l) of TiO$_2$-USNPs in water, at 0 hpf and 48 hpf, died immediately. Interestingly, at this concentration the pH of the suspension was around 3 which could explain the immediate expiration of the embryos. Exposure at 100 mg/l (47 m$^2$/l) on the other hand, delayed hatching of the embryos (which normally occurs between 2 - 3 dpf). This was possibly due to the impairment of hatching gland functions or hardening of the chorion by adsorption on the surface. The specific surface area of TiO$_2$-NPs at 1000 mg/l is about 50 m$^2$/l, which is comparable to 100 mg/l of TiO$_2$-USNPs. However, no such delay in hatching was observed. At 10 mg/l (4.7 m$^2$/l), TiO$_2$-USNPs did not have significant effects, in agreement with cytotoxicity results observed in vitro at the same concentration. Only injection of TiO$_2$-USNPs caused significant malformations, especially in the form of pericardial edema, possibly due to their uptake inside the cells during embryogenesis. Hyperspectral imaging showed the localization of TiO$_2$-USNPs in the heart as well as near the brain after injection at 0 hpf. In these embryos an overexpression of MYO1C was observed. This unconventional myosin is required for zebrafish glomerular development and its down regulation has been associated with pericardial edema in zebrafish embryos.

In summary, this study showed the in vivo potency and anti-angiogenic effect of TiO$_2$-USNPs.

Paper IV. The effects of ultra-small TiO$_2$ nanoparticle and single walled carbon nanotubes on endothelial cells: next generation sequencing and transcriptome sequencing (RNA-seq) analysis.

In order to understand the mechanism behind the anti-angiogenic effect of TiO$_2$-USNPs, in this study the endothelial cells were studied at the transcript level. The analysis was done at two exposure time points, namely 1 h (to decipher the initial impact of NPs) and 24 h. Endothelial cells were exposed to none toxic concentration (10 mg/l) of TiO$_2$-USNPs (5 m$^2$/l), TiO$_2$-NPs (0.5 m$^2$/l) and SWCNTs. The analysis was performed using transcriptome sequencing (RNA-seq).

In general TiO$_2$-NPs caused the most differential expression at both time points, followed by TiO$_2$-USNPs and SWCNTs. TiO$_2$-NPs and TiO$_2$-USNPs shared the most common transcripts, followed by TiO$_2$-NPs and SWCNTs. Only two transcripts were common to all exposures at 24 h. Most of the transcripts for cells exposed to TiO$_2$-USNPs and TiO$_2$-NPs were up-regulated.
while for SWCNTs they were mostly down-regulated. After 1 h most of the
differentially expressed transcripts in cells exposed to TiO$_2$-USNPs, (also in
common for TiO$_2$-NPS exposures) coded for mitogenic proto-oncogenes
such as FOS, JUNB and FOSB, as well as Early growth response protein
1(EGR1). These are induced by various extracellular signals such as, cyto-
kines and environmental stress, and vascular injury, and act as regulators of
cell proliferation, differentiation, and transformation.

After 24h, the exposure to TiO$_2$-USNPs induced the over expression
of transcripts involved in lipid and cholesterol metabolism. Also, some genes
were involved in ECM organization which could indicate shear stress.

In addition to FOSB, TiO$_2$-NPs induced transcriptional regulation of
markers of inflammation, suggesting a higher toxicity response compared
to TiO$_2$-USNPs. The transcripts were involved in cellular senescence, ER
stress and UPR responses. After 24 h, the differentially expressed transcripts
were involved in cholesterol metabolism, UPR/ER stress and heat shock
responses.

SWCNTs exhibited a more unique response, that is, inducing the differen-
tial expression of several non-coding transcripts. Most of transcripts were
also down regulated. After 24 h the differentially expressed transcripts were
also involved in ER stress/UPR response. However, none of the transcripts
were involved in lipid or cholesterol metabolism.

In summary, the effects of SWCNT can be attributed to early signs of
oxidative stress. While the induction of transcripts involved in lipid and cho-
lesterol metabolism in TiO$_2$-NPs, can be explained by ER stress. The mecha-
anism behind the induction of transcripts involved in lipid and cholesterol
metabolism in cells exposed to TiO$_2$-USNPs require further investigation.

Paper V. Assessment of the safety of functionalized iron oxide NPs in vitro:
introduction to integrated nano-impact index.

The effect of exposure to NPs is normally reported in a case-by-case
manner, but an integrated nanoimpact scale has not been developed yet. The
aim of this study was to develop INI based on classical toxicological end-
points to function as an early warning scale of nano-impact. In this paper, we
compared the calculated INI for each SPION as a percentage of the total
effects and arbitrarily defined the non-impact for INI: 0-10 as low impact,
>10-40 as medium impact, and >40 as high impact. As a proof of concept,
the effect of SPIONs functionalized with TSC, PEI, APTES, and chitosan
were assessed on human keratinocytes and endothelial cells i.e. representing
primary and secondary routes of exposure. The effect of surface coating, cell
type and initial cell culture density in toxicity assays was also evaluated. The results showed that although the keratinocytes cell culture medium influenced some oxidative activity, nonetheless, none of the SPIONs produced intracellular ROS or superoxide. The initial cell density of the cells (especially endothelial cells) greatly impacted toxicity results. Core-SPIONs (no surface coating) and APTES-SPIONs were genotoxic in both cell lines, while TSC-SPIONs were only genotoxic in endothelial cells. Endothelial cells were more sensitive to all the SPIONs showing similar medium INI values (17-26 %) with PEI-SPION and TSC-SPIONs having the highest and lowest INI value. In the case of keratinocytes on the other hand, only PEI-SPION had severe effects, with an INI of almost 80 %, while the other SPIONs had similar negligible INI (all < 10 %). We can therefore conclude that PEI-SPIONs were the most potent SPION in the cell types studied.
7. Discussion and concluding remarks

Experimental considerations

As with all scientific research, the study of the biological effects of NPs is not without challenges. Some experimental considerations that I also encountered during my PhD study should be taken into account when performing nanotoxicological studies.

Firstly, a comprehensive characterization of NPs is essential. The exposure environment affects the physicochemical properties of NPs. Therefore NPs should be characterized before, during and even after exposure in order to fully understand changes in their properties. For instance, aqueous medium components may affect the types and concentrations of ROS generated by NPs as it was observed in paper II and other studies for ZnO-NPs. This is related to the electronic structures of NPs and the redox potential of the different ROS generation reactions. Also, in paper I a change in the surface charge of USNPs was observed, possibly due to the presence of amino acids in the cell culture media. Moreover, in order to perform a more physiologically relevant study, the effects of aging, light, temperature and environmental impurities need to be taken into account during characterization and exposure.

Secondly, assessing correct dosage during in vitro assays is another important aspect to be considered. The dose of the NPs is often reported as either mass per volume or specific surface area. These values, however, far from indicate a realistic exposure concentration, especially for cells in the culturing flask exposed to NPs. Since in in vitro assays cells are normally cultured at the bottom of a culture plate, exposure to the NPs suspended in the media will not be homogenous as they aggregate and sediment on top of the cells. Other factors such as solubility, purity, and heterogeneity in size of the NPs, can also impact exposure dose.

The possible interference of NPs with classical toxicity assays is also important to consider. For example, it has been shown that NPs affect assays applying colorimetric/fluorometric measurements, or that SWCNTs interfere with the MTT assay. Therefore results should be confirmed by several assays in order to draw correct conclusions. Conversely, the choice of appropriate biological test systems, as well as exposure time and method, are all essential in determining relevant nanoimpact.
Omics-technologies enable addressing important questions such as species-specific toxicity, and low-dose effects. Still, since the biological pathways are often highly regulated and interconnected, no single omics approach can provide a holistic view of the cellular impact. For example, genomics and transcriptomics analysis do not provide information about the effects observed due to post-translational modifications (PTM).

Finally, there is a need for efficient approaches that allow linking physicochemical, molecular and cellular level responses with whole organism and population responses. For this purpose, the adverse outcome pathway (AOP) was established\textsuperscript{235,236}. The use of AOP in nanotoxicological studies is not free from limitations as it will require identification of the key links between responses, as well as key biomarkers at different levels of biological organization. Nevertheless, the application of targeted AOPs could offer a powerful approach to collect, organize and generalize information.

INI, as proposed in paper V, represents another method to integrate responses. This method is still under development. The biggest challenge in the development and application of INI are: 1) to integrate the induction and inhibition effects with an equal value in the total impact, 2) to consider the variability and complexity of the biological response; and finally 3) to normalize, and correlate of the individual units per parameter and the response. So far, we have compared the calculated INI for each NPs as a percentage of the total effects and arbitrarily defined the nanoimpact. The next step is to define appropriate controls and establish values for the nanoimpact.

We suggest that complementing the AOP of NPs with an INI value could provide a predictive tool for nanotoxicological studies. In the near future, nanotoxicology could turn into nanosafety by filling our gaps with methods to measure, anticipate, and even predict the effects of exposure to NPs and their consequences for human health and the ecosystem.
Figure 16. Adverse outcome pathway and suggested methods for assessment of each step in nanotoxicology. Image adapted from Ankley et al.236.
Summary of the toxicity of NPs

The results obtained from the study of CuO-NPs, Ag-NPs, ZnO-NPs and SPIONs agreed well with findings in the literature. In summary, CuO-NPs caused the most cytotoxicity both through release of ions and ROS. In yeast cells, they induced intracellular LDs production, while in hepatocytes they caused the decrease of membrane lipids and HMGCS1 involved in cholesterol metabolism.

Ag-NPs were the second most cytotoxic NPs, yet we could not detect oxidative activity or ion leaching. Still, possibly due to their small size, they could disrupt cellular ultra-structure in yeast, decrease SM and increase the total cellular lipid amount. Their effect on the proteome was similar to CuO-NPs, i.e. affecting those involved in metabolism reflecting a cell in an increased demand of energy.

ZnO-NPs were not cytotoxic. In yeast cells the excess ions produced were stored inside large vacuoles. In hepatocytes cultures they exhibited oxidative activity which in combination with Zn ions may have contributed to the effects observed in the proteome. Specific impact on proteins involved in signaling as well as on nuclear proteins was observed. They were the only NPs without impact on lipids. Due to their large size, it is possible that their cellular uptake and subsequent toxicity were limited.

Endothelial cells were more sensitive to all the SPIONs compared to keratinocytes. The surface coating of the NPs affected the biological impact of SPIONS with PEI-SPIONs and TSC-SPIONS being the most potent and biocompatible NPs respectively in both cell types.

Interestingly SWCNTs did not cause severe cytotoxicity, genotoxicity or cellular impact despite producing the highest amount of ROS. However, they induced differential expression of genes involved in stress responses, possibly as a sign of early oxidative and ER stress. Also, they did not affect angiogenesis or embryos in vivo.

TiO$_2$-NPs (30 nm) were not cytotoxic but were genotoxic and had no adverse effects in vivo. Transcriptomics analysis points however to a more toxic response, namely ER stress after 1 h of exposure followed by induction of differential expression of transcripts involved in cholesterol metabolism and heat shock response after 24 h exposure. It is therefore interesting that in both SWCNTs and TiO$_2$-NPs, these early stress responses did not hinder angiogenesis. Since TiO$_2$-NPs did not induce intracellular ROS production, the mechanism behind their ER stress response requires further investigation.
Toxicity and biological impact of TiO$_2$-USNPs

The effects from exposure to TiO$_2$-USNPs were the most intriguing, especially due to their unique properties and limited information regarding their biological impact. Therefore, here I will combine data from all the studies (paper I-IV) in order to provide an overview of their effects. The impact of TiO$_2$-USNPs was studied on different biologic systems, starting with a unicellular organism (S. cerevisiae), followed by different in vitro cell cultures i.e. primary cells (hepatocytes) and endothelial cells, and finally scaling the complexity to 3D cell culturing assessing angiogenesis, and in vivo using zebrafish embryos.

After characterization in each study, we could conclude that the effects observed were due to their size and direct interaction with the cells. This provided a way to study nanotoxicity without the effect of oxidative stress and solubility.

In summary, TiO$_2$-USNPs were not cytotoxic in vitro (only exhibiting mild cytotoxicity in yeast cells). They are taken up by the cells and localize inside vesicles, but can escape and gain access to the DNA, and were thus genotoxic.

Hepatocyte lipidomic analysis showed a decrease in the SM of the lipid rafts, while proteomic analysis showed the elevated levels of proteins in the electron transport chain and thus pointing to an increase of cellular energy demand. Transcriptomic analysis of endothelial cells exposed showed a specific increase of transcripts involved in the lipid and cholesterol metabolism. TiO$_2$-USNPs were also anti-angiogenic both in vitro and in vivo. It is unclear whether this specific impact on lipid metabolism could hinder angiogenesis.

At high concentrations (1000 mg/l), they cause total mortality of zebrafish embryos due to acidifying the water. The reason for this acidification is unclear, but as described earlier it might be due to the effect of curvature on attached components or possibly enhance water splitting. At 100 mg/l they induced delay in hatching, however at 10 mg/l they did not cause toxicity agreeing with their in vitro effects at the same concentration. Upon injection they cause malformations, especially pericardial edema.

Using this data, an AOP scheme can be obtained (figure 18) to provide a general overview of the effects of TiO$_2$-USNPs. However, the association between effects observed at cellular levels to those at the organism level would require additional investigations, for example performing proteomics analysis on endothelial cells and in vivo models.
Figure 17. AOP of TiO$_2$-USNPs. ETC: electron transport chain, SM: sphingomyelin, NO: nitric oxide.
8. Sammanfattning

Användning av nanopartiklar (NPs), dvs partiklar mellan 1-100 nm, i olika industrier såsom färg, textil och läkemedel har lett till betydande framsteg inom dessa områden. Storleken gör att NPs per viktenhet får en mycket stor yta och därmed kan få olika optiska, magnetiska eller elektroniska egenskaper. På senare tid har intresset växt för tillämpning av ultra-small NP (USNPs) vid 1-3 nm på grund av deras unika egenskaper som avviker från fria molekyler och större NPs. Parallellt med dessa framtidslöften, finns dock en växande oro för säkerheten av NPs, eftersom deras storlek är jämförbar med celler och proteiner. Risken finns att NPs kan orsaka skador i det organ som exponeras, till exempel lungor eller hud. NPs kan även komma in i blodomloppet där de kan orsaka störningar som kan leda till hjärt- och kärlsjukdomar som åderförkalkning och högt blodtryck. Dessutom kan de via blodcirkulationen föras till andra organ såsom till exempel lever och framkalla sjukdomar. Det huvudsakliga målet med denna avhandling var att undersöka säkerheten och bakomliggande mekanismer till toxiciteten av ett urval av metalloxid NPs samt USNPs. Vi fokuserade mest på exponeringseffekter på lever och kärlsystemet. Genom att välja rutilformen av titandioxid (TiO$_2$-USNPs) som inte producerar reaktiva syreföreningar (ROS), kunde vi studera biologiska effekter som orsakades endast på grund av deras storlek och direktkontakt med cellen. Vi visade att TiO$_2$-USNPs orsakade DNA-skador men inte celldöd. De var angiogeneshämmare och inducerade uttryck av gener som är involverade i lipid- och kolesterolomsättning i endotelceller. I hepatocyter å andra sidan, leddes det till överuttryck av proteiner i elektrontransportkedjan och minskning av lipider i cellmembranet. Zebrafiskembryon som exponerades för höga koncentrationer av TiO$_2$-USNPs i vatten dog, medan lägre koncentrationer orsakade försening i kläckning. TiO$_2$-USNPs kunde även medföra missbildningar när de injicerades i embryo. Större TiO$_2$-NPs (30 nm) var också genotoxiska men hade inga effekter varken på eembryon eller på angiogenes. Dock inducerade de uttryck av gener som är inblandade vid stress i det endoplasmatiska retiklet (ER) samt kolesterolmetabolism. Detta tyder på en mer toxisk respons i cellerna jämfört med TiO$_2$-USNPs. Muromgärdade kolnanorör (SWCNT) producerade mest ROS bland de NPs vi studerade, men de var inte allvarligt toxiska. De inducerade även uttryck av gener som är involverade i tidig ER-stress. NPs av kupferoxid (CuO-NP) var de mest toxiska NPs vi studerade. Denna toxicitet uppstod på grund av både jonavgivning och ROS-produktion, och påverkade lipidmetabolismen i cellerna. NPs av silver (Ag-NP) var också cytotoxiska och orsakade störningar i cellkomponenter och lipider. ZnO-NP var inte cytotoxiska men ökade storleken på vakuoler i jästceller. Slutligen, studerade vi superparamagnetiska järnmetabolism (SPIONs) med olika beläggningar. Därefter använde vi oss av en matematisk modell för att utveckla ett nanopåverkansindex (INI) som ett verktyg för att möjliggöra jämförelse av nanotoxikologiuppgifter.
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