Investigation of nanoparticle toxicity: Characterization of protein corona and evaluation of oxidative stress by protein carbonylation

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ERKLÄRUNG

Hiermit versichere ich, die vorliegende Arbeit mit dem Titel „***Investigation of nanoparticle toxicity: Characterization of protein corona and evaluation of oxidative stress by protein carbonylation***“ ohne Benutzung anderer als der zugelassenen Hilfsmittel selbständig angefertigt zu haben. Alle angeführten Zitate sind als solche kenntlich gemacht. Die vorliegende Arbeit wurde in keinem früheren Promotionsverfahren angenommen oder als ungenügend beurteilt.

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3. **Drießen, M.D.,** Luch, A., Haase, A., 2014. Protein carbonylation as a marker of oxidative stress induced by nanoparticles: Analysis of 16 inorganic nanoparticles. NanoSafety Cluster "Nanosafety Forum for Young Scientists". Syracuse, Italy: 08.-09.10.2014.

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KURZZUSAMMENFASSUNG

Die fortdauernde Entwicklung neuer Nanopartikel (NP) mit verschiedenen Eigenschaften (Material, Oberflächenmodifikationen, *etc*.) erfordert eine Priorisierung dieser Materialien in ihrer Gesundheitsbewertung. Dafür werden zuverlässige Screening-Methoden benötigt.

Für eine Klassifizierung oder Gruppierung müssen toxikologische Wirkungsweisen ebenso wie die Charakterisierung der Materialien hinsichtlich intrinsischer und systemabhängiger („*in situ*“) Eigenschaften betrachtet werden, wobei auch letztere wiederum die biologische Wirkung der NP beeinflussen. Die Proteinkorona z.B. bestimmt die Interaktion von NP mit Zellen. Das Agglomerationsverhalten hat über die Sedimentation der NP Einfluss auf die Dosis, welche die Zellen erreicht.

Daher befasst sich der erste Teil dieser Arbeit mit dem Einfluss von Oberflächenmodifikationen auf die systemabhängigen Eigenschaften von NP. Der Schwerpunkt lag auf oberflächenmodifizierten SiO_2 und ZrO_2 NP. Die Oberflächenladung hat großen Einfluss auf das *in situ* Verhalten der NP , bestimmt die Bindung von Surfactantproteinen der Lunge und die Interaktion mit lipidhaltigen Medien. Qualitative und quantitative Analysen der Proteinkorona zeigten, dass die Proteinadsorption von Material, Oberflächenmodifizierung und Inkubationszeit abhängig ist. Die Ergebnisse erlaubten zum ersten Mal eine direkte Korrelation zwischen der Charakterisierung *in situ* und von *in vivo* Daten einer Inhalationsstudie an Ratten mit denselben NP. Hier zeigten sich Korrelationen zwischen der Proteinadsorptionsgeschwindigkeit und den einzelnen Entzündungsparametern in der Lunge, sowie zwischen der NP-Lipidinteraktion und der Lungenbelastung *in vivo*.

Oxidativer Stress ist ein häufig für NP-Toxizität identifizierter Mechanismus. In dessen Folge können reaktive Sauerstoffspezies entstehen, welche wiederum direkt oder indirekt mit Proteinen reagieren. Sie können diese auch carbonylieren. Daher lag der Fokus im zweiten Teil der Arbeit auf der Untersuchung solcher Proteincarbonyle. Zum einen wurde ein Screening-Ansatz verfolgt, zeitgleich aber auch die Untersuchung toxikologischer Wirkmechanismen auf Basis der Proteincarbonyle. Ein breites Spektrum von NP wurde in NRK-52E Zellen mittels Carbonylspezifischem 1D/2D-Immunoblot untersucht. Hier fanden sich extrem hohe Korrelationen zwischen Carbonylierung und Viabilität (84%) sowie

Oberflächenreaktivität der NP (83%). Die Identifizierung der carbonylierten Proteine ließ einen ersten Einblick in mögliche Wirkmechanismen zu, während die statistische Auswertung der NP-spezifischen Carbonylierungsmuster eine erste Gruppierung erlaubte. Insgesamt zeigte sich, dass diese Methode neben der Verwendung als Screening-Ansatz auch für Einblicke in Wirkmechanismen und damit den Aufbau von „*Adverse Outcome Pathways*“ nützlich ist.

Der hier generierte Datensatz ist daher eine gute Basis für die Entwicklung von Gruppierungsansätzen.

ABSTRACT

The constant development of new nanoparticles (NPs) with different properties (size, material, coating, *etc*.) necessitates a priorization in hazard assessment, which in turn requires reliable screening methods.

Classification or grouping of NPs however requires consideration of toxicological modes of action as well as a NP characterization including intrinsic and system dependent ("*in situ*") properties. The latter in turn are able to influence the NPs' biological effect. The protein corona (PC) for instance governs the interaction of NPs with cells while the NP agglomeration behavior may influence their sedimentation and thereby the particle dose reaching the cells.

Based on these concepts, the first part of this thesis describes the influence of surface modifications on the system dependent properties of NPs. It is focused on modified SiO_2 and, ZrO_2 NPs. While surface charge is a major influencing factor, NP lipid interactions were mainly governed by the binding of lung surfactant proteins. Qualitative and quantitative analysis of the PC showed that the formation depended on chemical composition, surface modification as well as on time of incubation. The study allowed for the first time a correlation between *in situ* results and the results of an *in vivo* short time inhalation study in rats using the same NPs. We found that the speed of PC formation was correlated with the observed inflammatory reactions in the rat lungs, while NP lipid interactions were correlated with the lung burden observed *in vivo*

Oxidative stress is one cause for NP toxicity. One result of oxidative stress is the presence of reactive oxygen species in the cell, which can react directly or indirectly with cellular proteins and thereby form carbonyls. Thus, the second part of the thesis focused on the analysis of protein carbonyls. Here, a broad spectrum of NPs was tested for the induction of protein carbonylation in the NRK-52E cell line using a 1D/2D-immunoblot approach. Very high correlations between carbonylation and cell viability (84%) as well as surface reactivity (83%) were found. A proteomic identification of the carbonylated proteins allowed a first insight into possible toxicity mechanisms. The NP specific carbonylation patterns were used for statistical evaluation, which allowed a first grouping of the used materials.

In summary, it was concluded that the protein carbonylation analysis is suitable as a screening method supporting prioritization. Identification of carbonylated proteins

can even be used for classification or grouping approaches. Moreover, the ability to gain insights into modes of action is of great value, as this might enable the deciphering of adverse outcome pathways and could be used in predictive toxicology.

The dataset presented here is therefore an excellent starting point to unravel nanomaterial grouping strategies.

ABBREVIATIONS

1D/2D	one/two dimensional
AlOOH	γ-AlOOH, aluminum oxide hydroxide, (boehmite)
AgNP/AgNPs	silver nanoparticle/s
AUC	analytical ultracentrifugation
BALF	Bronchoalveolar lavage fluid
$BaSO_4$	barium sulfate
CCM	complete cell culture medium (cell culture medium, usually supplemented with 5-20% serum, glutamine and often antibiotics and/or antimycotics)
CeO_2	cerium dioxide (ceria)
DCF	dichlorofluorescein
DCS	differential sedimentation centrifugation
DLS	dynamic light scattering
ESR	Electron spin resonance
FCS/FBS	fetal calf serum/fetal bovine serum
LC-MS	liquid chromatography-mass spectrometry
MALDI-MS/MS	Matrix assisted laser desorption ionization mass spectrometry
(MW)CNT	(multi walled) carbon nano tubes
NP/NPs	nanoparticle/s
NM/NMs	nanomaterial/s
NTA	Nanoparticle tracking analysis
nS	native surfactant
PC	protein corona
PEG	polyethylene glycol
PS	polystyrene
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SiO_2	silicon dioxide (silica)
TiO_2	titanium dioxide, (titania)
UV	ultra violet
WST	water soluble tetrazolium
ZnO	zinc oxide
ZrO_2	zirconium dioxide (zirconia)

CONTENTS

1 Introduction

1.1 Nanoparticles

Nanotechnology is a fast-growing economic area. The number of nanotechnology based products and processes has been growing rapidly during the last years (see also chapter 1.1.1). With the increasing use of nanoparticles (NPs) the scientific interest to also address potential adverse health effects has increased, as can be seen by rising numbers of publications.[1-3]

Scientists have been working either wittingly or unwittingly with materials at the nano level for hundreds of years. However, only recently a formal definition of the term nanomaterials (NM) emerged. According to ISO/TS 27687 2008 all materials with at least one dimension or components/structures in the range of 1 -100 nm are NMs. Furthermore, this definition differentiates between nanostructured materials (*e.g.* polymers containing nano-objects) and 3 major types of nano-objects: Nanoplates, nanowires, and nanoparticles (NPs), with 1, 2 or 3 dimensions in the size range respectively (Figure 1-1).

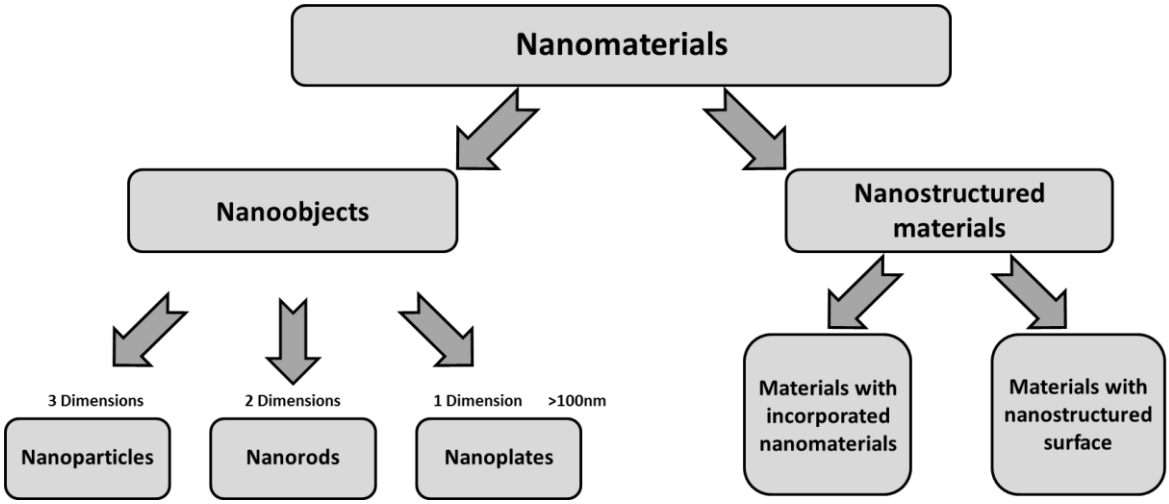

Figure 1-1 Schema of types of nanomaterials according to ISO/TS 27687 2008

NPs can consist of almost every material. They can be produced naturally or by intentional synthetic processes. Natural NMs are often produced during events like volcano eruptions, forest fires, sandstorms, and other natural phenomena.

Synthetic NPs are accessible via two main paths: the bottom-up-procedures, usually starting with single atoms, ions or small molecules; and the top-down-procedures which often starts with bulk materials that are disintegrated into smaller parts. Particularly the bottom-up-procedures can lead to NPs in terms of composition, size and shape that are tailored to specific uses. One aspect of this is the use of different surface modifications or coatings, which help to stabilize dispersions and keep particles as separate as possible from each other. Coatings usually employ either steric hindrance (*e.g.* (bio-)polymers) or electrostatic repulsion.[4] While NP can of course be synthesized to be stable without a specific coating (usually marked as unmodified/uncoated/naked/*etc*.) even these particles are never truly 'clean' (refer to chapter 1.2 and chapter 3.1).

The small size of NPs leads to unique properties, which also cause specific physical, chemical and biological (resp. toxicological) effects, which include an extremely high surface to volume ratio or possibly a very high reactivity and even quantum effects.[1,2] Most of the chemical and biological effects are usually attributed to the specific volume and the specific surface area which often lead to a much higher reactivity than the respective bulk materials.[5] Furthermore, when NPs enter a biological environment, their system dependent ("*in situ*") properties differ significantly from their intrinsic (i.e. purely physical or chemical) properties. One very prominent example is the interaction of NPs with bio-molecules such as proteins and the properties of the thereby formed corona (refer to chapter 1.2) which in some cases can significantly change the behavior of NPs in biological environments (from biol. media, *via* cells to tissues and animals).[6]

In this context knowledge about the respective influence of parameters like *e.g.* size, material, surface coating and charge, crystallinity, *etc*. on nano–bio interactions is still lacking. To create datasets that can be reliably used for grouping or even predictive toxicology, approaches which focus on the correlation between intrinsic properties and their influence on the system dependent (or *in situ*, meaning in the biological test environment, *e.g.* cell culture medium) behavior of the NP are needed. In addition, research on modes of actions of NPs, are needed to gain a deeper understanding of nanotoxicology. This may also support the discovery of biomarkers and enable new toxicological endpoints.[1,7] To achieve this, studies focusing on complex sets of particles, i.e. systematic modifications of a few factors (like charge, composition or coating) are needed.

Preferably these studies should also include both extensive characterization of NPs as synthesized (intrinsic properties) and several biological endpoints. This knowledge would not only help to develop predictive methods, but would also support the development of reliable and NP specific screening methods as well as safe-by-design concepts of NP synthesis.

In This work sets of 16 (chapter 3.2) and 25 (chapter 3.3) NPs were used. Both sets comprised 4 SiO_2 and 4 ZrO_2 NPs which were available as unmodified, basic, acidic and polymer modified versions (Figure 1-2). Here the surface coating was modified while keeping the other parameters identical too specifically study the influence of surface modifications on *in situ* characterization and on toxicology.

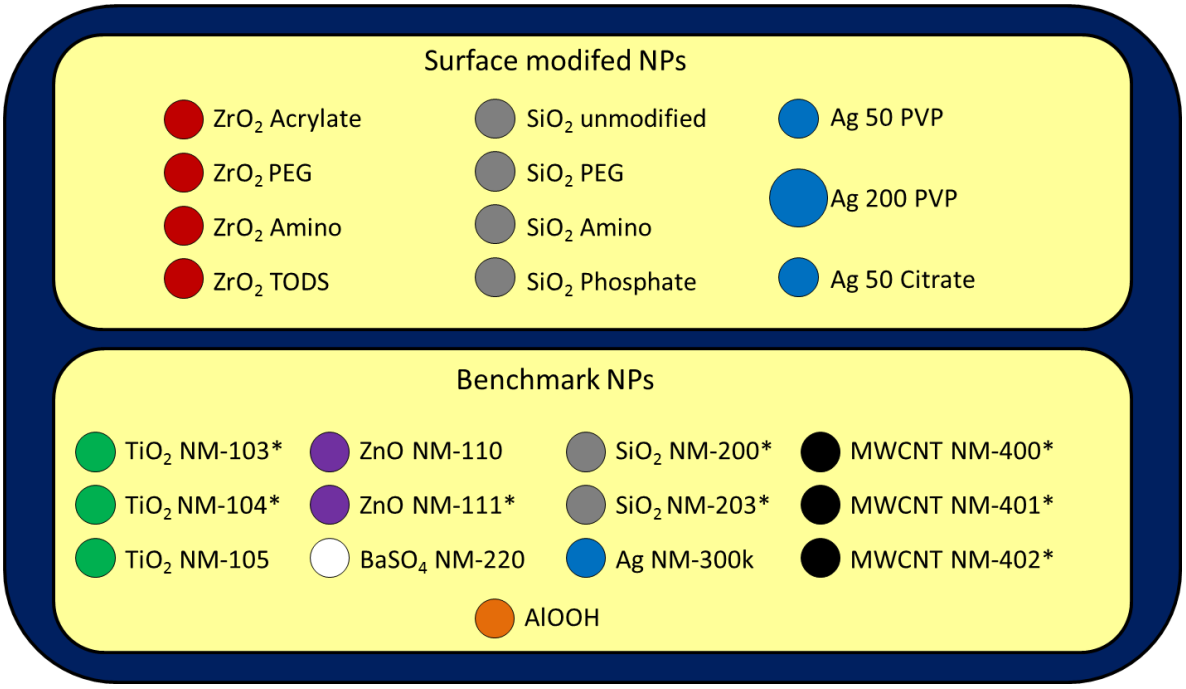

Figure 1-2 NPs used in this thesis. Surface modified materials are shown in the top half, benchmark particles are shown in the lower half. NPs marked with an asterisk (*) were only used for the study presented in chapter 3-3.

Moreover three Ag NPs in two sizes and two coatings were used (smaller polymer and citrate coated particles as well as larger polymer coated particles). In addition particles comprising further SiO_2 materials, TiO_2, ZnO, AlOOH and $BaSO_4$ were used as either positive (showing an toxicological effect) or negative (no toxicological effect) *'benchmark particles'*. Benchmark NPs are very well described in terms of their toxicology and physicochemical properties. As virtually no

reference NPs for toxicology are available, the use of such benchmark particles is important to enable comparison of toxicological datasets gathered in *e.g.* different projects. Therefore particles were chosen that have for example been used by several European and international projects. The work presented in this thesis focuses on the characterization (chapter 3.2) of the modified particles, as synthesized and under conditions used for *in vitro* testing and in native surfactant (a model for conditions encountered *e.g.* in animal lungs). In the final part (chapter 3.3) the focus is on the study of modes of actions of nanoparticles.

1.1.1 NP applications

Due to their unique properties the field of applications for NPs has been growing in recent years. Especially consumer products such as cosmetics, textiles, packaging, colors and paints, but also the field of nanomedicine (*e.g.*: contrast agents, drug carriers) and novel materials (*e.g.* carbon nanotube containing plastics) recently gained major attention from researchers and industry.[1,3,8-14] The major types of industrially relevant NPs can be discerned: Metal (*e.g.* Ag), metal oxide (*e.g.* TiO_2) and carbon based (*e.g.* (MW)CNTs) NPs, while in nanomedicine also polymer NPs have gained importance.

Metal NPs made from *e.g.* gold, palladium or platinum are used in the chemical industry for catalytic processes, as they can improve the efficiency of chemical reactions due to their larger specific surface area.[5,15] Ag NPs have for example been used in textiles, as additives in polymers or as coatings to reduce fungal and bacterial growth.[12,16] They can also be found in electronic applications, *e.g.* as a printing ink for solar panels and circuits.

Metal oxide particles like nano-TiO_2 are often used in cosmetics such as sunscreen (UV-filter), but also soaps and toothpaste (usually as an abrasive agent).[17,18] It has also been used as a coating to promote sterility of medical tools.[16] Also ZnO NPs are often applied in sunscreen as UV-filter, but also because of their antibacterial properties in crèmes.[1,18,19] Nano-ZrO_2 can for example be used in self-cleaning stoves (as a destructive catalyst). SiO_2 NPs find application as fillers (*e.g.* in paints and polymers) that increase scratch resistance, but also (usually as micro/nano mix) as anti-caking agents and glidents in powdered foods.[18,20]

Carbon based NPs like (Multi-walled) carbon nanotubes ((MW)CNTs) are *e.g.* used as conductors (heat or electricity), but also to lighten and strengthen alloys and polymers.[1,8,18,21] However, in biomedical application also polymers (or polymer finished core shell structures), lipid vesicles and micelles are used.[10,13,22,23] .

1.1.2 Potential routes of exposure to NPs

There is an ongoing discussion about tentative harmful effects of NPs on the general public. Therefore, the interaction of NPs with biological entities such as cells, tissues, organs or bodies is highly investigated. Especially knowledge of absorption, distribution, metabolism and excretion (ADME–concept), respectively their kinetics in human or animal bodies is of importance in this context.[24]

Potential uptake pathways are essentially inhalation, oral uptake and skin penetration. Particles can be taken up to the respective primary target organs (lungs, gastro intestinal tract and skin respectively), from where NPs can potentially travel be translocated to secondary target organs (*e.g.* liver, kidneys, spleen). Especially due to the use of NPs in cosmetics, like TiO_2 and ZnO in sunscreen, skin penetration has been intensely studied. However, most studies show that healthy skin is an impenetrable barrier for NPs.[25-27] As more and more NPs are applied in food or food contact materials, uptake by ingestion has to be considered as well.[28] While it is often stated that the largest part of ingested NPs passes the gastro intestinal tract quickly and are mainly excreted with the feces,[29] several studies found a distribution to secondary organs after oral uptake.[24,30,31] This can be explained by considering bio-soluble and insoluble NPs. Insoluble NPs (*e.g.* CeO_2, Ir) pass through the body relatively unhindered. However, while exact uptake pathways for soluble NPs could often not be elucidated, it was shown that *e.g.* Ag and SiO_2 NPs can be dissolved in the gastro intestinal tract and later reform inside or even outside the gastro intestinal tract.[28,32,33] While the above uptake pathways are important, inhalation has proven to be the main pathway of concern, due to increased intentional and unintentional exposure.[29] In the literature, particle sizes (measured as mass median aerodynamic diameter) between 1 µm and 3 µm are reported to be the optimal range for deep lung (alveoli) deposition. Particles smaller than 1 µm are immediately exhaled to a large percentage (~80 %). Of the remaining particles again only a fraction is actually deposited in the alveoli.[34] This fraction however is usually reported to be mostly in the nano-range (smaller than or about 100 nm). However the acquired lung burden (the amount of deposited particles) rather seems to depend not only on the material and size, but also on the treatment concentration, as Landsiedel *et al.*

reported lung burdens ranging from 2.7 % (SiO_2 Phosphate) to as high as 18.8 % (ZrO_2 Acrylate) of the total treatment dose.[35] However, of this total amount of deposited particles, only a fraction can then possibly reach the blood and thereby distribute to the rest of the body.[34,36,37] Kreyling *et al.* used radiolabeled Ir NPs (15 and 80 nm) to treat rats *via* an endotracheal tube. They described a size dependent uptake, yet only fractions smaller than approximately 4 % (15 nm Ir NPs) of the lung burden were found in the complete carcass of the rats.[38-40] The main route of NP removal from the lungs is the alveolar clearance, which mostly proceeds by phagocytosis of the deposited particles by alveolar macrophages. These travel to the mucociliary escalator, which can transport NP carrying macrophages as well as bigger particles to the larynx where they are finally excreted from the lung (they can however enter the oral route if swallowed).[7,29,34] However, some NPs can damage the macrophages after ingestion, which can lead to oxidative damage of tissue, and (secondary) deposition of NPs, as well as inflammation, which in turn can lead to fibroses and tumorigenesis.[5,34,36,41-44] This has been discussed for carbon black in particular.[41,42] Another aspect is the possible translocation of NPs into immune cells and from there to the lymphatic system, where they are deposited. From the lymph nodes it can then be possible for NPs to reach other secondary organs *via* the blood.[7,29,34] One special case of exposure to NPs *via* inhalation is the NP uptake *via* the olfactory nerve into the brain, and thereby bypassing the blood-brain-barrier.[29,45] While this is a possible scenario, uptake via the lungs is considered to be much more probable,[29]

1.1.3 Cellular uptake

The biological effects of NPs on the cellular level are mostly determined *via* attributes like size, charge, surface modifications and chemical composition. Cellular uptake has been shown to proceed via active pathways (*e.g.* endocytosis, phagocytosis),[46] but also passive uptake like diffusion is discussed. Inside the cell NPs can reach various organelles such as lysosomes, mitochondria, the nucleus *etc*. and might cause different types of toxicity depending on their localization.[20,46-52] For example positively charged particles like polymer coated CeO_2 disrupted lysosomes,[53] while ZnO NPs located in lysosomes releases Zn^{2+} which lead to a destabilization of the lysosome.[54] While SiO_2 NPs (43 nm) were assumed to cause direct damage to the mitochondrial membrane in a hepatocellular carcinoma cell line,[55] several other materials (*e.g.* Ag, TiO_2) were shown to induce swelling of mitochondria.[47] In any case disruption of the mitochondria lead to increased ROS levels in the cell. Nanoparticle presence in the nucleus is usually linked to genotoxicity, often by ROS generation.[47,52]

While research on biological effects of NPs has multiplied in recent years, knowledge about biokinetics of NPs as well as their cellular modes of actions is still lacking.

1.2 Bio-nano interactions: The protein corona*

It is commonly accepted that the comprehensive characterization of NPs is the basis for any toxicological (or biological) study.[52,56-60] Yet, there is an ongoing discussion about which parameters are essential to understand the physiological interactions of NPs and whether intrinsic (as synthesized) or *in situ* (in a biological environment) properties of NPs determine (these) interactions.[61] Due to the nature of solid particles in aqueous solution, surface interactions with the surrounding media control the general state of NPs in dispersion. Therefore, any physical or chemical change of the NP surface should influence its interactions, such as the formation of a biomolecule corona in biological media. These changes would in turn provoke differences in the aggregation/agglomeration behavior and thereby influence the effective dose of test subjects, such as cells or tissues. Ultimately this will have effects on commonly studied parameters, such as cellular uptake and biodistribution and hence influence toxicity.[6,62-68] Therefore, a complete NP characterization should also include data on the behavior of NPs in relevant biological media. In this context it is of great importance to consider the biomolecule – or, more specific, the individual protein corona (PC) that NPs form in different media.[59,69,70]

In general no surface can ever be completely clean, but it is always covered in molecules present in its environment. Thus, it seems obvious, that also no particle can be considered to be 'purely' its core material. As the NP surface is the interface between solid and surrounding medium a thin, layer of molecules will form here. Using NPs in any biological (or toxicological) setting a specific medium in which the NPs are transferred is always present. Such media can range from cell culture media in *in vitro* experiments to complete plasma or other, organ specific fluids like *e.g.* pulmonary surfactant.[71]

As NPs are commonly dispersed prior to use, particularly for *in vitro* experiments, the principle holds true: The NPs (with or without coating) exposed to a complex environment will, upon first contact, begin to coat with constituents of this particular environment (the Vroman effect).[72] These constituents could either be

* This chapter is considered to be a short introduction into the topic, as the protein corona and its effects have been described in detail in the review–styled book chapter, chapter 3.1 of this thesis.

solvent molecules or biomolecules ranging from amino acids, sugars, lipids, to whole peptides and proteins. Especially biomolecules will influence the interaction of the NPs with biological entities like cells or tissues. This is reflected in the hypothesis that the NP-protein complex is, "*What the cell 'sees' in bionanoscience*" as was stated by Walczyk *et al.*.[65] While for a long time the PC was the major focus of research, recently the small biomolecule corona has received additional attention.[71,73] As the surface, or rather the outermost layer of molecules of a NP defines its interaction with any biological entity; the formation of a PC on the NP surface supposedly changes the interaction of biological systems (especially cells) with the particle (Figure 1-3).

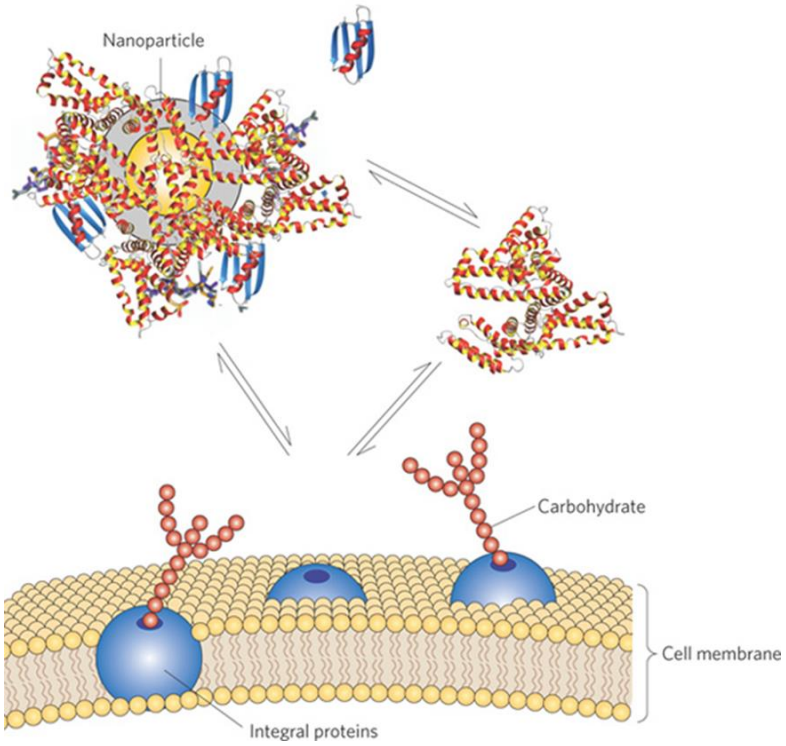

Figure 1-3 NP protein corona complexes in interaction with a cell. Upon contact with a biological medium NPs form a protein corona, that is changed over time. The so called 'hard corona' is able to influence the interaction of NP and cell. The figure is reprinted from [74].

A recent publication by Ge *et al*. reviews the altered effects in detail.[71] The most obvious changes were reported for NP uptake into the cells as well as for overall toxicity. NP uptake is usually considered as a two stepped active process, where the first step is the binding of the NP to the cell surface and the second step is the internalization.[46] It has been shown for example that adsorption of corona covered silica (50 nm) or PS-NP (40 and 100 nm) to the cell membrane of A549

cells is significantly lower than that of bare particles.[46,63] The same is true for internalization of corona carrying PS-NP in A549 cells. This was shown either in presence or absence of serum.[46,63] The same study also showed that the cytotoxicity of silica NPs is higher when cells are exposed to the bare particle.[46,63,71,75] These studies further indicated the possibility that the corona might influence the cellular localization of NPs after uptake.[63] For further examples refer to chapter 3-1.

Even though the concept of proteins (and other biomolecules) binding to synthetic materials immediately after entering a biological environment has been introduced by L. Vroman, 53 years ago,[72] this phenomenon has been studied mainly with focus on medical implants and devices.[76] Recently the NP protein corona being a special case of these interactions has gained more and more attention from researchers. While some principles are transferable,[65,68,71,73,77] NP protein interactions are not well enough understood yet. [71,73,77,78] One of these principles is the fact that NPs form a PC by binding to highly abundant proteins directly after being transferred into a protein rich environment. This binding has been shown to be very fast.[79] After initial formation of the PC, loosely bound proteins may be exchanged with proteins that have a higher affinity to the surface of the respective NP.[79] This process is also known as the Vroman effect.[76] This has been published to occure within 24 – 48 hours.[79,80] However, other studies suggest that the corona formation is already completed after a few minutes.[81] This PC formation leads to a shell of proteins bound so tightly to the NPs, that they will not (or only very slowly) dissolve again by washing or even after isolation and redispersion of the NP-PC complex in a completely protein free environment (Figure 1-4).

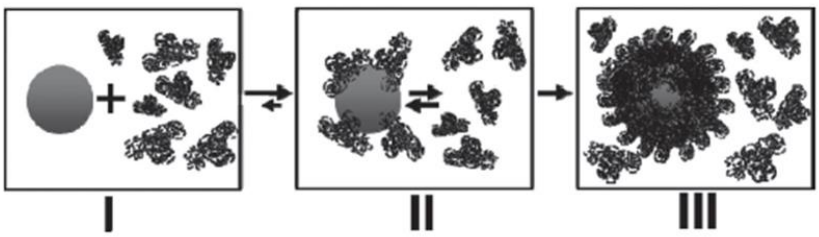

Figure 1-4 Interactions between NP and proteins. When a NP enters a biological medium (I) it starts to interact with high abundant proteins (II) and ends up with a protein shell that is no longer easily soluble (III). Figure reproduced from [80].

The NP-PC complex, that can be experimentally isolated and studied, is usually referred to as the “hard” corona. In contrast, proteins bound loosely to the NPs (or in turn to the proteins of the PC) are referred to as the “soft” corona, which is virtually inaccessible by experiment, as it desorbs during the NP-PC isolation. Still, it has been shown that the “hard” PC can change and evolve while the NP-PC complex is trafficking through different environments (*e.g.* uptake into cells/tissues/*etc*.).[82] This would leave a fingerprint of proteins on the NP that is specific for the various environments it has passed through. Several parameters determining the pattern and species of proteins adsorbed to the NP surface have been identified. These parameters include mainly physicochemical properties. Regarding shape and size (or surface curvature), it has for example been shown by Lundqvist *et al.* that polystyrene (PS) NPs of 50 nm and 100 nm while carrying the same coating do form coronas with a markedly qualitative difference. While several high abundant proteins were commonly shared, especially low abundant proteins made up the unique coronas.[83] In contrast Tenzer *et al.* reported a rather quantitative difference in the protein coronas around SiO_2 NPs ranging from 20 nm to 100 nm in diameter, in which they identified the same set of 125 proteins in all coronas, but found differences in the amount bound.[84] Other parameters like chemical composition, coating and with it the surface charge (zeta potential) as well as hydrophobicity along with the incubation time have been shown to have an effect on corona composition.[74,77,85-87] In addition, parameters of the surrounding medium (*e.g.* pH, protein and salt concentration, *etc*.) also have a large impact.[77] This can be explained, when taking into account that the forces involved in corona formation are mainly considered to be van-der-Waals and electrostatic interactions along with hydrophobic and charge effects and of course steric considerations.[88] The latter concept is for example used when polymer chains like polyethylene glycol (PEG) are used to limit NP protein interaction.[89] If NPs are stabilized by charge alone, binding proteins can, in some cases, overcome and neutralize the charge, which would lead to increased agglomeration.[90] In contrast however, it has been shown that NPs lacking stability will disperse much better after being coated with proteins like serum albumin.[91]

This demonstrates that the NP-PC depends on the composition of the physiological medium in which it is formed. Relevant uptake routes of NPs include specific exposure scenarios, which distinguish between intentional uptake in

nanomedicine (especially intravenous injections, but also oral and inhalation) and accidental uptake by *e.g.* consumers or production workers (wounds, inhalation and oral). These scenarios allow defining a set of biological fluids that are of special interest with regard to studying the NP-PC complex. While blood (or rather plasma/serum) is the medium connected with injection, lung lining fluid is the first barrier after inhalation and gastric fluids correspond to ingestion. However, the situation is quite different with respect to *in vitro* cytotoxicity studies, which are usually carried out in serum containing complete cell culture medium (CCM). Here, usually the cell culture medium is supplemented with 5-20% v/v of serum (usually fetal bovine serum).

Blood plasma or serum NP-PCs have often been studied due to their physiological relevance,[84,85,92,93] Also several studies exist for CCM, as the usual medium in cell culture experiments.[46,71,77,94] While recently also lung lining fluid (or native surfactant, BALF) has received more attention in this field, there are not many studies published so far.[52,95,96]

Several methods can be applied to either analyze NP changes in the medium (*in situ*) or after isolation of the NP-PC complex (*ex situ*). These methods for PC analysis have been part of recent reviews [77,97,98] as well as chapter 3.1 of this thesis.[60] One general strategy is the use of methods that determine size and agglomeration state of NP-PC complexes, as the average size of particles will change when a layer of proteins is adsorbed to their surface. As reviewed by Walkey *et al.* the increase in diameter can be quite significant.[89] For example 50 nm SiO_2 NPs showed a corona thickness of 26 nm, and values as high as 35.3 nm were reported for 200nm sulphonated polystyrene NPs.[87,89] Methods commonly used to study changes in size include dynamic light scattering (DLS),[65,99] nanoparticle tracking analysis (NTA), analytical ultracentrifugation (AUC),[100] and differential sedimentation centrifugation (DCS).[65,98,101] These methods often do not require reisolation of the NP-PC complex, but can be used to determine changes directly in the medium. Another approach is the identification of the PC composition. Here, usually the NP-PC complex needs to be isolated (*e.g.* by centrifugation). Methods that are mainly used to identify proteins attached to the NP surface are usually based on liquid chromatography resolution followed by mass spectrometric analysis (LC-MS) techniques, which use either gel-free approaches or rely on an additional separation step to partition proteins or

peptides by an additional property such as the size or the isoelectric point (*e.g.* by 1D/2D-SDS-PAGE).[82,84,85] This is however not a final list of methods used to analyze NP protein interactions, but rather an overview, as more details are given in chapter 3.1.

1.3 Cellular effects of NPs: Oxidative stress

Oxidative stress has become the predominating paradigm to explain NP toxicity. It is closely linked to general toxicity as well as specific effects like inflammation and DNA damage.[102-107]

Oxidative stress is a direct result of a cellular imbalance between antioxidants and reactive oxygen species (ROS). ROS are generated on the one hand as byproducts of the respiratory chain and cellular reactions that require oxygen (mostly leakage of reactive species).[108] On the other hand ROS can result from other (xenobiotic) sources, like chemicals or NPs. For NPs several reactions are discussed.[1] Mainly surface reactions driven by excitation via UV-light, Fenton-like reactions, and catalytic chemistry either on NPs or dissolved ions are discussed, yet also desorption of bound ROS on the NP surface can add to the total ROS load (Figure 1-5).[1]

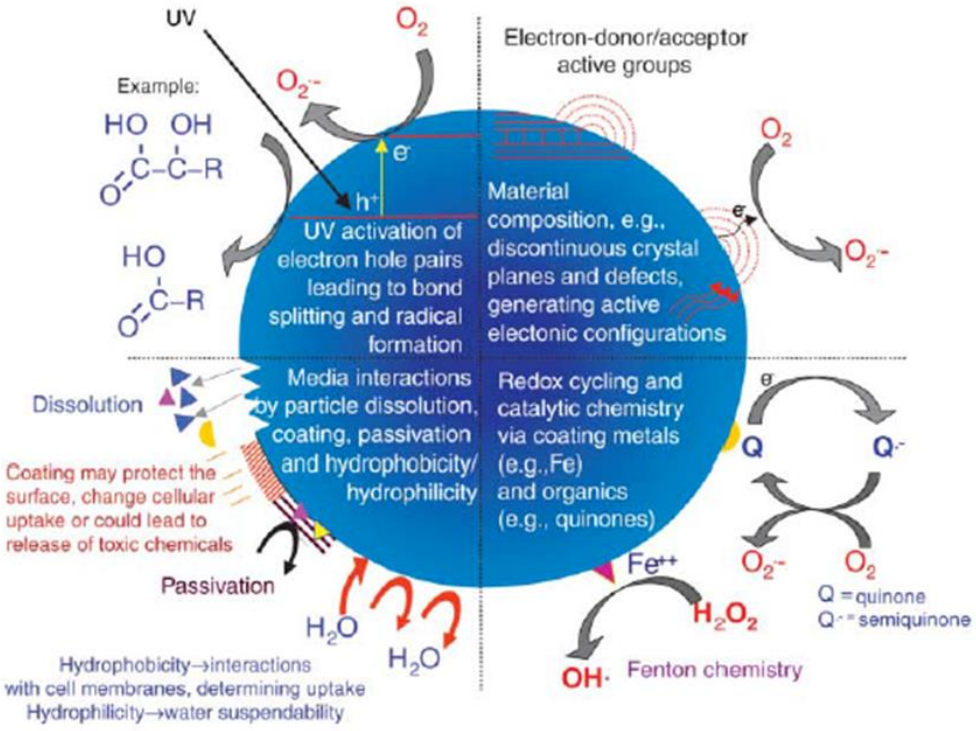

Figure 1-5 Possible mechanisms by which nanomaterials interact with biological tissue. Several examples are illustrated that highlight different aspects of the NP composition and their influence on biological reactions with a focus on ROS generation. The figure was reproduced from Nel *et al.:* 'Toxic potential of materials at the nanolevel', *Science* 2006.[1]

The type of ROS generated depends on the NP material and it's biological environment. This could be shown by electron spin resonance using (ESR) chemical detection probes for superoxide radicals and singlet oxygen (1-hydroxy-3-carboxy-pyrrolidone: CPH) on the one and for hydroxyl radicals (5,5-dimethylpyrrilidone N-oxide: DMPO) on the other hand.[109]

Compared to bulk materials the ratio of surface area to material mass is significantly increased for NPs. As size decreases, the surface area, and thereby the number of exposed atoms, increases. The amount of ROS is most probably dependent on the surface area, as was shown by studying silver NPs (Ag NPs), where 15 nm particles showed a drastic increase in ROS levels compared to 50 nm particles at the same mass dose.[110] It has also been shown that particles not showing ROS generation in a cell-free environment can still lead to ROS generation inside cells.[111] Xia *et al.* presented an example for different NP based effects taking place either at the same time or consecutively.[111] The authors found a biphasic progression of ROS generation, the first wave being caused by the disruption of phagosomes by NPs, followed by increased leakage from damaged mitochondria as part of the apoptotic cycle.[111]

Similarly to these secondary effects, it was found that, especially in cells of the immune system (macrophages) the uptake of NPs can lead to the so-called respiratory burst,[112,113] which usually is part of the defense against biological stressors such as bacteria or viruses,[114,115] and releases ROS as well as cytokines from the macrophages. If this effect is continuous it may also lead to oxidative stress or damage in neighboring cells, which in turn can lead to inflammation and a higher likeliness of tumorigenesis on the tissue level.[116] A final effect may be a possible depletion of antioxidants in cells (*e.g.* glutathione), which could sensitize cells to a level where normal amounts of ROS lead to oxidative stress.[117] Usually classical dye-based methods like dichlorofluorescein (DCF), which are based on the dye being directly oxidized by cellular ROS, are used to screen for ROS formation. While several assays are universally used and accepted in classical toxicology, several setbacks in nanotoxcicological applications have been discussed. As one often used example, DCF, like many other dye-based assays suffers from NP specific interferences, ranging from NP absorption/fluorescence at equal wavelengths, plasmon resonance to the possible

adsorption of dyes.[104,118] Another possibility to overcome almost all of these problems is to detect ROS not directly, but indirectly via measuring protein oxidation. This feasible and promising alternative for NP screening and research on modes of action in nanotoxicology was also used in this thesis (chapter 3-3).[119] Protein oxidation (carbonylation) along with lipid and DNA oxidation is one of the results of oxidative stress. Depending on the system, up to 70% of cellular ROS react with proteins.[120,121] Amino acids like methionine, cysteine and tyrosine are most sensitive to oxidation and thus often targeted in redox-proteomics studies.[122] The (often reversible) oxidations (and corresponding reduction) at the sulfur groups of cysteine and methionine are part of cellular signaling mechanisms. In contrast formation of protein carbonyls is an irreversible process. Depending on its final location, the introduction of a carbonyl group can break protein structure on all levels (*i.e*. primary, secondary or tertiary structure). Disruption of the protein structure often leads to aggregation and loss of function. Carbonylation can also occur naturally due to free ROS, and is one way for a cell to mark proteins for proteolysis.[123] Some studies also suggest a link to signal transduction pathways.[124] Protein carbonylation is in any case the predominant type of oxidative damage to cellular proteins.[123] The oxidation of proteins (insertion of aldehydes or ketones) can happen via multiple pathways: Directly by reaction with a ROS either at the backbone, leading to a fragmentation of the protein, or at one of the amino acid side chains (usually arginine, lysine, proline or threonine) as well as indirectly by reacting with an active oxidation product of cellular small molecules (lipids, sugars, *etc*.), usually at the side chains of lysine, histidine or cysteine (Figure 1-6).[125]

These reactions have in common that either an aldehyde or ketone is introduced into the protein, which can subsequently be utilized for detection of the damaged proteins, by derivatization with molecular ‘tags’ like biotin hydrazide or 2,4-dinitrophenylhydrazine (DNPH). These in turn can be used for a very specific detection *via* antibodies[126,127] or protein precipitation.[128] As the chemical modification of carbonyls is usually done after cell lysis and protein isolation, usually no significant amount of NP remains in the sample that could influence the procedure. Moreover the detection step occurs after NP removal from the sample, preventing it from NP specific effects.

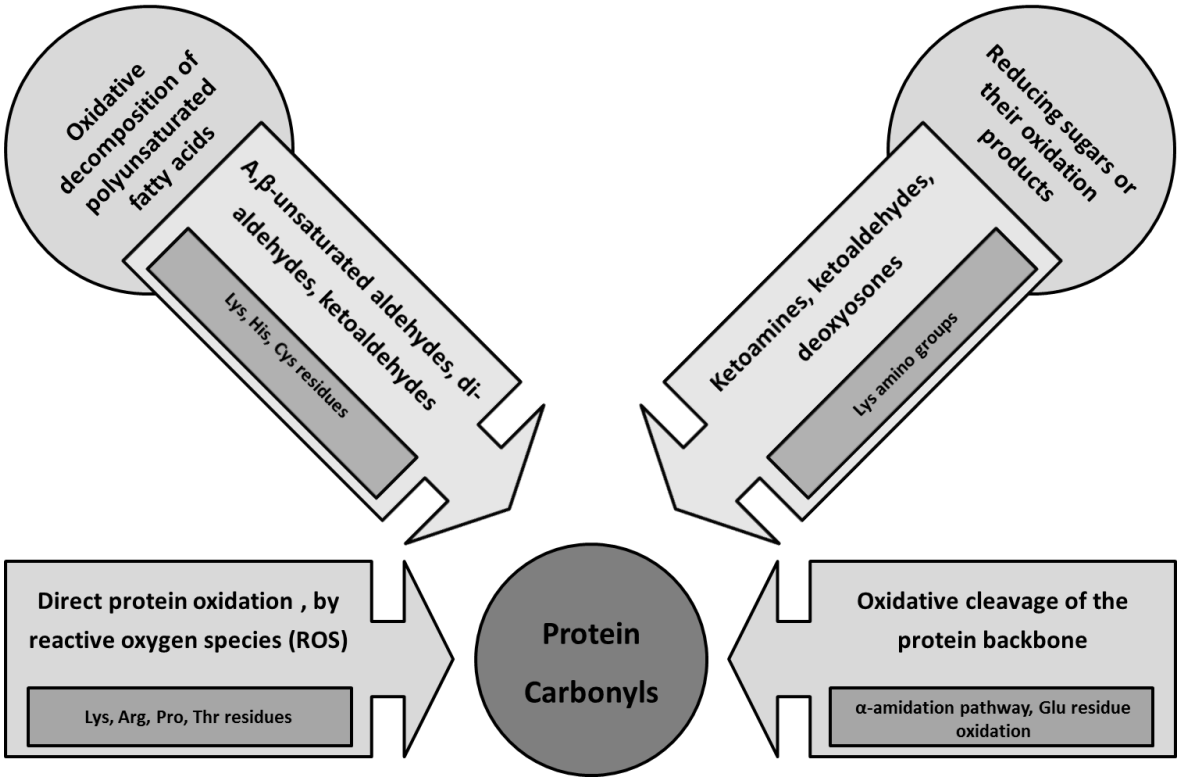

Figure 1-6 Pathways to carbonylated proteins. Proteins can be oxidized either directly by ROS or indirectly by reacting with oxidizing products of *e.g.* lipids or sugars reacting with ROS. Figure modified after [120].

The analysis of protein carbonyls has long since been used in studying aging and its effects, as especially anti-oxidative capabilities fail over time and the steady-state ROS level in cells could rise.[129,130] There are also studies that link higher levels of protein carbonyls to neurodegenerative diseases. Here the majority of oxidized proteins belong to metabolic pathways like glucose metabolism, mitochondrial function and protein degradation, but also structural proteins were found.[131] Another link was made to metabolic diseases, where a higher general level of carbonyls has been associated with *e.g.* insulin resistance[75,120,132]or obesity in humans[75] and mice.[133]

However, in toxicology also the effects of small molecular xenobiotics on carbonyl levels have been studied. Especially chemicals that disturb the electron transport chain (*e.g.* rotenone, paraquat, diquat) lead to higher ROS levels and consecutive protein carbonylation.[125] Also insecticides[134] and fungicides[135] have been shown to increase carbonylation in black tiger shrimp and mice respectively. Yet also

every-day toxins like ethanol have been shown to specifically increase carbonylation of betaine-homocysteine S-methyl transferase in rat liver.[136]

Recently protein carbonylation has also been studied in nanotoxicology, where they are used either as biomarker for oxidative stress in mussels and other aquatic species or to study modes of NP actions.[119,137-140] One example is a study showing that AgNPs but not Ag-ions induced protein carbonylation in *daphnia magna*.[141] Other studies showed effects in THP-1 or mixed neuronal cells, where ROS induction, cytotoxicity and protein carbonylation were caused by AgNPs, but not by gold NPs.[48,142] Also an AgNP size dependency in protein carbonylation in human colon epithelial cells[143] and macrophages was reported.[110]

While the concept of protein carbonylation is not new, studies focusing on it are still few in nanotoxicology. However, it seems that the analysis of protein carbonylation could provide an approach for NP screening and priorization (*e.g.* in hazard assessment), as well as grouping along with more information about modes of actions, as was shown by the recent study which is part of this thesis (chapter 3.3).[119]

2 Aim of the work

This work addresses two associated objectives. The first objective was to test the effects of NP surface modification on the *in situ* properties. Special emphasis was laid on the interaction of NPs and proteins in different biological media (CCM, native surfactant (nS)) and whether the results could be linked to toxicological outcomes. Another aspect was to provide a thorough characterization of the surface modified materials, which were then intensely studied in the second part of the thesis with respect to their toxicology. The second objective aimed at the use of protein carbonylation, which results from oxidative stress, to develop a reliable *in vitro* screening approach. To this end a rat kidney cell line was used to assess protein carbonylation, which was compared to other methods that may be used to assess oxidative stress potential such as intracellular ROS generation, and NP surface reactivity as well as to overall cytotoxicity. In a subsequent step carbonylated proteins were identified to test whether this approach is useful to unravel NP toxicity mechanisms.

3 Publications and Manuscripts

3.1 Analyzing the biological entity of nanomaterials characterization of nanomaterial properties in biological matrices

Christian A. Ruge*, Marc D. Driessen*, Andrea Haase*, Ulrich F. Schäfer, Andreas Luch, and Claus-Michael Lehr

*These authors contributed equally to this work

This chapter was published on 03. December. 2014:
Chapter 4 (pp.59-95) in the book "*Safety of Nanomaterials along Their Lifecycle: Release, Exposure, and Human Hazards*", edited by W. Wohlleben, T.A.J. Kuhlbusch, J. Schneckenburger and C.-M. Lehr, CRC Press, Taylor & Francis Group, Boca Roton, FL, USA
(ISBN: 9781466567863)
Link to eBook:
https://www.crcpress.com/Safety-of-Nanomaterials-along-Their-Lifecycle-Release-Exposure-and-Human/Wohlleben-Kuhlbusch-Schnekenburger-Lehr/p/book/9781466567863

The Authors contributions:

As indicated above, equal contributions to this review–styled book chapter. Contributions focused on NP characterization *in situ* and in serum / serum-containing cell culture medium.

3.2 Influence of agglomeration and specific lung lining lipid/ protein interaction on inhalation toxicity

Wendel Wohlleben[*], Marc D. Driessen*, Simon Raesch, Ulrich F. Schaefer, Christine Schulze, Bernhard von Vacano, Antje Vennemann, Martin Wiemann, Christian A. Ruge, Herbert Platsch, Sarah Mues, Rainer Ossig, Janina Tomm, Jurgen Schnekenburger, Thomas A. J. Kuhlbusch, Andreas Luch, Claus-Michael Lehr[§*], Andrea Haase[§*], 2015 submitted

*These authors contributed equally to this work, [§]Corresponding authors

This chapter was submitted to Nanotoxicology

This chapter was published on 17 March 2016 under a modified title:
Influence of agglomeration and specific lung lining lipid/protein interaction on short-term inhalation toxicity
Wohlleben, Driessen et al., Nanotoxicology, 10(7):970-80
DOI: 10.3109/17435390.2016.1155671
Link: http://dx.doi.org/10.3109/17435390.2016.1155671

The Authors contributions:

DLS analysis of all NPs
Protein corona resolution (2D-SDS-PAGE)
Protein corona identification (MALDI-MS/MS)
Sample preparation for LC-MS identification
Analysis of proteomic results (2D-SDS-PAGE & MS)
Discussion/evaluation of results (with other authors)
Preparation of manuscript (with other authors)

3.3 Proteomic analysis of protein carbonylation: A useful tool to unravel nanoparticle toxicity mechanisms

Marc D. Driessen, Sarah Mues, Antje Vennemann, Bryan Hellack, Anne Bannuscher, Vishalini Vimalakanthan, Christian Riebeling, Rainer Ossig, Martin Wiemann, Jürgen Schnekenburger, Thomas A. J. Kuhlbusch, Bernhard Renard, Andreas Luch and Andrea Haase**

This chapter was published on 02.November 2015:
Driessen *et al.*, Particle and Fibre Toxicology, 12:36
DOI: 10.1186/s12989-015-0108-2
Link: http://dx.doi.org/10.1186/s12989-015-0108-2

The Authors contributions:

DLS analysis of al NPs
Cytotoxicity testing (JRC Particles)
Analysis of protein carbonylation by 1D/2D immunoblot
Protein identification by MALDI-MS/MS
Pathway analysis
Data evaluation/discussion
Preparation of manuscript
.

4 Summary and Conclusion

It is a widely accepted supposition that a complete and extensive characterization of NPs is the fundamental starting point for toxicological studies and understanding the observed effects.[144] As the rationales behind this characterization are reviewed extensively in chapter 3.1 it should suffice to state that the sometimes dramatic changes (*e.g.* agglomeration/aggregation, dissolution, protein corona formation) NPs can undergo when transferred from an abiotic to a biological environment . However, these changes currently can not be predicted reliably enough when purely based on intrinsic properties.

Therefore approaches, like the one suggested by Sayes and Warheit[59] include characterization throughout their life-cycle and on several levels:

1st) "as synthesized" (usually in powder state, encompassing basic physical parameters like chemical composition, size, *etc.*);

2nd) characterization of abiotic dispersions (*e.g.*: aggregation/agglomeration, dissolution, reactivity, *etc*.)

3rd) characterization in the relevant biological environment or "*in situ*" (*e.g.* protein/biomolecule corona formation, aggregation/agglomeration.

This approach is reflected by the approach presented in the first part of this thesis (chapter 3.2).

The study presented in chapter 3.2 focused on the influence of surface modifications on the *in situ* characterization of a set of NPs. This includes investigations to reveal whether certain *in situ* parameters, could be correlated to an *in vivo* inhalation study performed with the same NPs. To determine this, the characterization of NPs was performed in several biologic fluids (lipid mixture, Curosurf™, nS and CCM).

In this study it was confirmed that, as expected, surface charge was a major determining factor of biomolecule (i.e. lipids and proteins) corona formation as well as aggregation for this set of NPs. It was observed that positively charged NPs showed a generally higher interaction with lipid containing media. Moreover protein and lipid adsorption in nS followed the same trend. Surprisingly, strong implications were found that binding of surfactant protein A (SP-A) to the NPs

facilitated the lipid binding which was unexpected, as earlier studies suggested the opposite. In CCM negatively charged NPs tended to attract the highest protein amounts. The protein coronas formed after 24h showed similarities based on the core material but were influenced by the surface modifications. A time dependent analysis of the corona revealed a stronger influence of the surface modification, as both SiO_2 unmodified and SiO_2 Amino showed rapid protein adsorption while SiO_2 PEG and SiO_2 Phosphate exhibited a progressive increase in the amount of bound proteins.

The results allowed for the first time to correlate two parameters of the *in situ* characterization to results obtained in an earlier *in vivo* study. On the one hand lipid affinity of SiO_2 NPs showed a good correlation with their deposition in the lung, on the other hand a very strong correlation between the speed of protein corona formation and of inflammatory reactions in rat lungs was found.

The study presented in chapter 3.3 focused on the analysis of protein carbonyls induced by NP treatment. While one key aspect was the influence of surface modifications on NP toxicity, also a large set of benchmark NPs was used. Another aspect was to determine whether protein carbonylation might be suitable for screening or even classifying NPs according to the biological response.

Levels of protein carbonylation as a result of oxidative stress caused by NP treatment of a rat kidney cell line (NRK-52E) was compared to intracellular ROS generation (DCF assay) and NP surface reactivity (ESR). While results of intracellular ROS detection showed only an agreement in 1/3 of the samples with either carbonylation or ESR, overlap between carbonylation and ESR as well as between carbonylation and cytotoxicity were in both cases higher than 80%. In total 11 out of 24 tested NPs caused higher levels of protein carbonylation. Applying a more detailed 2D immunoblot, unique carbonylation patterns were found for all NPs that induce carbonylation. Patterns of carbonylation by NPs of the same core material were influenced by the specific surface modifications. Statistical analysis of these unique patterns was performed and revealed clusters of materials that were in agreement with observed biological effects. Moreover, it was possible to identify a number of carbonylated proteins by mass spectrometry.

These could be used to gain first insights into possible modes of actions of the different NPs.
The results of the study showed that on the one hand analysis of protein carbonylation levels can be used as a predictive screening tool while, on the other hand a more detailed identification of carbonylated proteins can be a promising tool to gain insight into underlying mechanisms of NP toxicity.

It should be noted that the conditions for characterization of NPs in CCM used in chapter 3.2 are the same that have been used in the toxicological study presented in chapter 3.3 (*i.e.* time points, CCM composition). While it was possible to link certain aspects of the NP *in situ* characterization to biological (*in vivo*) outcomes in chapter 3.2, no specific parameters (other than surface reactivity) studied there could be directly linked to the results of the *in vitro* carbonylation in chapter 3.3. Obviously surface reactivity was linked to carbonylation, as several NP surface reactions (*e.g.* Fenton-like reactions) are discussed to cause ROS (refer to chapter 1.3). Surface reactivity should however be considered an 'as synthesized' parameter. In addition, it has to be pointed out that in literature very few, if any, NP collections can be found that offer a similar set of characterizations, both as synthesized and '*in situ*'. With respect to possible future implications – if protein carbonylation should be broadly accepted as a feasible screening method – the characterization information acquired for this rather large NP set can be invaluable, especially if new studies with different sets of materials are to be compared to existing results. The possibility to use protein carbonylation to identify modes of actions of NPs will provide much-needed knowledge.

Viewed in context of each other, one major conclusion can be drawn from the individual parts of this thesis: Both studies in chapter 3.2 and chapter 3.3 show that toxicological endpoints (*in vivo*, chapter 3.2; and *in vitro*, chapter 3.3) can indeed be linked to parameters of the NP characterization. Both studies agree that for the used set of NPs, NP core composition is the major determining factor, while the surface composition has a secondary, yet fundamental effect. However, it seems that the as synthesized surface (and thereby surface reactivity) rather than the protein or lipid corona is more influential on toxicity, compared to system-

dependent changes such as protein or lipid corona formation. Other influencing parameters such as size or crystallinity also play a role. However for the NP set used in this thesis those parameters seem to have subordinate effects.

Overall the work performed within the scope of this thesis revealed that linking NP *in situ* characterization to biological effects is possible, but not trivial. Due to the specific set of NPs used in this thesis, important insights into the influence of NP surface modifications on *in situ* characterization and toxicology were gained. Likewise, a new approach to NP screening and classification has been proposed. Taken together, the studies presented in this thesis provide a broad dataset, which allows the comparison of results, using the tested benchmark NPs as reference points, which will be of great value for future studies. Ultimately, data generated *via* this method might help to link material properties to the overall observed effects to build adverse outcome pathways, which in turn would expedite classification and regulation.

5 Outlook

From the work presented in this thesis several starting points for future studies emerge, which can either be based on the protein corona, or on protein carbonylation.

5.1 Protein corona based studies.

Even though the number of publications dealing with the protein corona is still rising, several aspects remain unclear. To gain a better general understanding of the corona effects on toxicology, correlations and other statistical approaches seem to be indispensable. For this a large amount of data is still available, yet most studies focus on a limited set of particles, which are additionally often model particles. In the future more studies focusing on “real life” NPs similar to the ones used in here in chapter 3.2 and including a very thorough characterization (both as synthesized and i*n vitro*) would be needed for a meaningful analysis.

Another aspect is the gap that still exists between the complex corona studied in *e.g.* serum or plasma where the focus lays mostly on the identification and quantification of binding protein species, and the study of single (or few) protein coronas, where the study of actual changes happening to the proteins is in the foreground. Here only carefully designed approaches can be successful. For example utilization of particles that form a corona of comparably low complexity and at the same time show toxic effect could be envisioned. Here a stepwise build up in complexity starting with only one or two proteins, at the same time comparing toxic effects might be a feasible approach.

Another question that is not nano-specific, but is maybe of even higher importance due to the discussed role of the protein corona is the following: How does the (*e.g.* serum) corona change in sera won from different species? As it is always the final goal to assess *in vivo* effects from *in vitro* tests the question often arises whether cell lines from *e.g.* rat would react differently when cultivated and exposed to NPs in rat serum, instead of fetal calf serum, as is usually the case. Studies testing this are apparently almost nonexistent. Combining this with the second part of this

thesis, a study also comparing the amount of stress induced between particles with coronas from different sera could be of interest.

5.2 Protein carbonyl based studies

The use of protein carbonylation as a screening method was proposed in the second part of this thesis (chapter 3.2). Even though already a panel of 25 NPs was studied there, much more information is needed. As was stressed before, all studies need to start with a thorough characterization. If this can be achieved and additionally several toxicological endpoints for a preferably large set of NPs can be studied in combination with protein carbonylation, the ability to use statistical grouping and prediction methods will be largely enhanced.

Another aspect that would warrant studying is the direct correlation between *in vitro* carbonylation and *in vivo* (carbonylation) effects. One could envision a complex study where animals and cell lines that can serve as models for certain organs (*e.g.* liver, kidney, lung, *etc.*) are treated under the same regime to allow a direct comparison. This might even allow to bridge the always present discrepancy between dosing *in vivo* and *in vitro*, as a conversion rate might be gauged from such a mirrored experiment.

Another type of study, more focused on the modes of actions of nanoparticles would be focusing on identifying even more of the carbonylated proteins *via* additional proteomic studies with an additional focus on effected cellular functions, comparing NP based effects with *e.g.* knockout cells, or using inhibitors. As a next step studies combining metabolomics and proteomic approaches assessing the *de facto* changes in metabolic pathways after carbonylation of several key proteins could be even more revealing.

6 Literature

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