A journey through the "nano" world - an analytical and toxicological challenge

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Fabian Lukas Kriegel

Die Dissertation wurde in englischer Sprache verfasst.

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Abstract

The rapid development of nanotechnology and its application in all areas of life not only provides many chances, but also poses potential risks for users and their environment. Modern health-based risk assessment strategies for nanomaterials include three major pillars: analytical data about surface chemistry, composition and size distribution patterns of manufactured nanomaterials (NMs), toxicological data from *in vitro* experiments and lastly also from *in vivo* experiments. This thesis provides scientific advancements for each of the three pillars, in order to enhance a holistic health based risk assessment approach for nanomaterials.

The chemical characterization, quantification and the determination of size distributions is a key issue in defining a NM and predicting its interactions. In the context of the research work of this dissertation, a further development of the single particle inductively coupled plasma mass spectrometry (sp-ICP-MS) technique is presented. With the help of this method the fast and reliable size distribution characterization of metal containing NMs is now possible. The new approach is based on the application of a dual sample introduction system using both, a pneumatic nebulizer and a micro droplet generator. The design of the system allows a comprehensive size characterization of NMs within 20 minutes. The obtained data is evaluated using three approaches presented in the thesis. Each of these approaches is based on a unique calibration principle. Thus, this work contributes to an independent and precise analytical characterization of NMs.

Furthermore, in order to gain insights into the solubility behavior of NMs after oral uptake a *in vivo* toxicological assessment was carried out. Aluminum-containing NMs (Al_2O_3 and Al^0) were administered to rats via oral gavage for three-days and the aluminum organ burden was determined. The comparatively low exposure level in combination with the ubiquitous aluminum background posed a challenge for conventional analytical techniques. We solved this problem in the present study by means of a matrix calibration and the application of a daily response factor, which enabled us not only to reliably determine the applied materials in organic matrices, but also to obtain information on the uptake and distribution of the two NMs. A special focus was

laid on the differences in the uptake of the NMs and the subsequent distribution of the materials in the intestinal and systemic organs (liver, kidney, spleen). Our work has shown that the materials behave significantly different, irrespective of similar size and composition. Especially shape and surface properties of the NM could be identified as decisive factors for these differences.

Moreover, *in vitro* experiments with the two aluminum containing NMs in human keratinocytes were carried out to further investigate the cellular uptake and intracellular distribution as well as the resulting metabolic changes within the cell. In order to make the exposure scenario more realistic and mimic ingredients of cosmetic products, the influence of the two bioactive vitamins A and D3 was tested in addition to the two NMs. The ICP-MS results showed significant differences between the uptake of the two NMs after addition of the vitamins. Time of Flight secondary ion mass spectrometry (ToF-SIMS) was then used to further investigate metabolic changes in the cell. It was found that the increased uptake of Al^0 NMs after vitamin D3 addition was due to a loss of cell membrane integrity. The combination of Al_2O_3 NM and vitamin D3 led to an increase in membrane stability. However, the exact difference could not be conclusively clarified in the study. Our results indicate the need for further research to identify which properties of a NM are decisive for the observed different interactions.

In summary, this work contributes to a more precise characterization and detection of NMs and to a better explanation of their interactions with biological systems. The pillar based methodology can be used for future research and thus contributes to a safer and more reliable application of nanotechnology without major health risks.

Zusammenfassung

Die rasante Entwicklung der Nanotechnologie und ihrer Anwendung in sämtlichen Bereichen des Lebens bietet nicht nur viele Chancen, sondern birgt auch Risiken für die Anwender und ihre Umwelt. Moderne Risikobewertungsstrategien für Nanomaterialien umfassen drei Hauptfelder: die physikochemische Charakterisierung von Nanomaterialien Durchführung von *in vitro*-Experimenten und abschließend auch *in vivo*-Toxizitätstestung. Diese Arbeit zeigt wissenschaftliche Fortschritte für jedes dieser drei Themenfelder, um den ganzheitlichen Risikobewertungsansatz für Nanomaterialien zu verbessern.

Ein zentraler Punkt bei der Definition eines NMs sowie für die Vorhersage von Interaktionen von Nanomaterialien mit lebenden Organismen stellt die analytische Charakterisierung der Größe dar. Im Rahmen der Forschungsarbeit dieser Dissertation wird eine Weiterentwicklung der single particle inductively coupled plasma mass spectrometry (sp-ICP-MS) Technik vorgestellt. Mit Hilfe dieser Methodik ist die schnelle und zuverlässige Größencharakterisierung von Nanomaterialien möglich. Der neue Ansatz basiert auf der Anwendung eines dualen Probeneintragssystems, welches sowohl einen pneumatischen Zerstäuber als auch einen micro droplet generator verwendet. Der Aufbau des Systems ermöglicht eine umfassende Erfassung der Partikelgrößenverteilung von Nanomaterialien innerhalb von nur 20 Minuten. Die erhaltenen Daten werden über drei in der Arbeit vorgestellte Ansätze ausgewertet. Jeder dieser Ansätze beruht auf einem einzigartigen Kalibrierungsprinzip. Somit trägt diese Arbeit zu einer unabhängigen und präzisen Charakterisierung von Nanomaterialien bei.

Um zu einer verlässlichen Risikobewertung von Nanomaterialien beizutragen, wurden die orale Aufnahme und Biokinetik von aluminiumhaltige Nanomaterialien (Al_2O_3 und Al^0) in einer 3 Tages Studie an Ratten getestet. Die vergleichsweise geringe Expositionsmenge in Kombination mit dem ubiquitären Aluminiumhintergrund stellte eine Herausforderung für die konventionelle Analytik dar. Wir lösten dieses Problem in der vorliegenden Studie mittels einer Matrixkalibration und der Einführung eines Daily Response Factors, wodurch wir nicht nur in der Lage waren, die applizierten Materialien in organischen Matrices verlässlich zu bestimmen, sondern auch Aussagen über die Biokinetik der beiden Nanomaterialien zu treffen. Ein

besonderer Fokus wurde dabei auf die Unterschiede in der Aufnahme der Nanomaterialien und der anschließenden Verteilung der Materialien im Darm und den analysierten Organen gelegt. Unsere Arbeit hat gezeigt, dass trotz ähnlicher Größe und Komposition eine signifikant unterschiedliche Verteilung der Nanomaterialien im Darm und den untersuchten Organsystemen vorliegt. Insbesondere die Form sowie die Oberflächenbeschaffenheit der Materialien könnten entscheidende Faktoren für diese Unterschiede sein.

Um die unterschiedliche Aufnahme und intrazelluläre Verteilung sowie daraus resultierende Veränderungen des zellulären Metabolismus zu erfassen, wurden humanen Keratinozyten mit den beiden aluminiumhaltigen Nanomaterialien exponiert. Um das Expositionsszenario etwas realistischer zu gestalten, wurden neben den Nanomaterialien der Einfluss von den beiden bioaktiven Vitaminen A und D3 getestet. Die ICP-MS Ergebnisse zeigten deutliche Unterschiede zwischen der Aufnahme der beiden Nanomaterialien nach Zugabe der Vitamine. Flugzeitsekundärionenmassenspektrometrie (ToF-SIMS) wurde anschließend eingesetzt, um metabolische Veränderungen in der Zelle näher zu untersuchen. Es zeigte sich dabei, dass die vermehrte Aufnahme von Al^0 Nanomaterialien nach Gabe von Vitamin D3 auf einen Integritätsverlust der Zellmembran zurückzuführen ist, während die Kombination aus Al_2O_3 NM und Vitamin D3 zu einer Erhöhung der Membranstabilität führte. Woran genau dieser Unterschied liegt, konnte im Rahmen der Studie nicht abschließend geklärt werden. Allerdings deuten unsere Ergebnisse auf einen weiteren Forschungsbedarf hin, um aufzuklären, welche Eigenschaften eines NMs ausschlaggebend für die beobachteten Interaktionen sind.

Zusammenfassend trägt die vorliegende Arbeit dazu bei, Nanomaterialien genauer charakterisieren und detektieren sowie ihre Interaktionen mit biologischen Systemen besser erklären zu können. Die vorgestellten Methodiken und Ansätze können für zukünftige Forschungsarbeit genutzt werden und damit zu einer sichereren und verlässlicheren Anwendung der Nanotechnologie ohne große gesundheitliche Risiken beitragen.

Abbreviations

AFM	atomic force microscopy
Ag	silver
Au	gold
CDE	caveolae-dependent endocytosis
CME	clathrin-mediated endocytosis
CRISPR-Cas9	clustered regularly interspaced short palindromic repeats
DAG	diacylglycerols
DLS	dynamic light scattering
EC	European commission
EELS	electron energy loss spectrum
EDX	X-ray energy dispersive spectroscopy
GSH	glutathione
ICP-MS	inductively coupled plasma mass spectrometry
LDPE	low-density polyethylene
MDG	microdroplet generator
NM	nanomaterial
NOAL	no adverse effect levels
NP	nanoparticle
NTA	nanoparticle tracking analysis
PA	phosphatidic acid
PC	phosphatidylcholine
PE	Phosphatidylethanolamine
PLA	D,L-polylactide
PN	pneumatic nebulizer
ROS	reactive oxygen species
SC	stratum corneum
SEM	scanning electron microscopy
sp-ICP-MS	single particle inductively coupled plasma mass spectrometry

SPR	surface plasmon resonance
STEM	scanning tunneling electron microscopy
TEM	transmission electron microscopy
ToF	time of flight
ToF-SIMS	time of flight secondary ion mass spectrometry
US EPA	United States Environmental protection agency
XRD	X-ray diffraction

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1. Introduction

Humanity can only exist through interaction with its environment, whether it is to feed oneself, to establish living space or to develop over time. Especially the advancement of new technologies is part of a developing civilization. [1] Solving problems through technological progress leads to new manufacturing processes and products. However, due to their novelty, these technological achievements are often associated with an uncertain health risk for manufacturers and users. [2] Examples of technologies where concerns about their safety arose only after market introduction include genetically modified organisms with the CRISPR-Cas9 system [3] or fracking [4].

Technologies that are considered questionable by the general public with regard to their security tend to spread slower and have considerable difficulties becoming widely accepted [1]. A comprehensive risk assessment using a “safe-by-design” approach could ultimately avoid negative implications for the user of a novel technology is therefore indispensable in order to guarantee a broad dissemination. This is the key to exploit the full potential of a technology. Nanotechnology is an example of a developing technology of the past decades, which is being discussed ambivalently due to its rapid and extended applications in various areas of daily life and also because of unknown risks and hazards for human health and the environment [5]. The following part of this work will deal with nanotechnology and NMs (NMs) in particular to elucidate not only the risks but also highlighting their applications.

In his lecture "There's Plenty of Room at the Bottom", the famous physicist Richard Feynman laid the foundation for today's nanotechnology. He described ideas and concepts on how new substances could be synthesized by manipulating individual atoms by nanoscaled machines [6]. Due to technical limitations at that time, Feynmann's ideas could not be translated into practical use right away. Finally, the invention of characterization techniques like the atomic force microscopy (AFM) [7] and scanning tunneling electron microscopy (STEM) [8] assisted significantly in turning Feynmann's vision into reality. Using advanced analytical technologies, the behavior of substances at the nanoscale could be investigated.

The term "nanoscale" usually refers to the size range between 1 and 100 nm, which corresponds to one billionth of a meter (10^{-9} m). The smaller an object becomes, the more the volume to surface ratio increases. Caused by the increased surface area, the number of surface atoms rises exponentially with decreasing size [9]. In order to illustrate this fact further, one should consider the following example: The proportion of atoms on the surface of a macroscopically visible solid is 10^{-7}, whereas a spherical NP (NP) consisting of 8000 atoms contains about 1600 atoms on the particle surface. As a result of the increased amount of surface atoms, the physicochemical properties of the NP changes. By reducing the size of substances to the nanoscale, not only their actual size changes, but also quantum physical effects occur more frequently. The cumulative influence of these effects leads to novel chemical, physical, optical, and mechanical properties of the NM [10].

1.1. Nano - the definition of something very small

Despite the benefits also risks may be associated with the frequent use of nanotechnology in modern day consumer products. However, a worldwide and over all regulatory fields commonly used definition of "nanoparticle" or "nanomaterial" is still missing. *Boholm et al.* reported 36 definitions for these two terms [11]. This illustrates the struggle of finding a comprehensive definition. The problem which policy makers are facing is a feasible definition of a NM in order to foster beneficial nano-based products but also to prevent potential hazards. Since the regulation of products which are not defined is not possible, the EU took action and presented the definition of a NM in 2011 as follows:

"A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %. By derogation from the above, fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm should be considered as NMs" [12].

The European commission (EC) aimed to develop a science based definition. In contrast to the definition of a NM from the United States Environmental protection agency (US EPA) [13, 14], the EC's definition does not differentiate whether a novel property is present due to the nanosize or not. The example shown is not the only difference in the various definitions. Some legislators distinguish whether a product is nanoscale in all three spatial dimensions and only then regulate it, while for others one spatial dimension in the nanorange is already sufficient. Aggregate size, composition and other characteristics also differ in the various definitions [15].

Despite this diversity of definitions, a comparatively large number of them include the term nanoscale or even an explicit size range [16]. However, such a strict size limitation could lead to hazardous particles not being properly regulated. Several scientists argue that a "one size fits all" regulation is not appropriate for the NMs cosmos. Instead, a regulation adapted to the class of the material is needed [17, 18]. This is a plausible approach as the following example will illustrate.

A 40nm gold (Au) NP is considered to be inert [19]. However, if the size of the Au particles is reduced to less than 3 nm, the particles become much more reactive. This is also the reason why 3nm Au NPs are used as catalysts [20]. According to almost all definitions, the inert particle would be regulated in the same way as the reactive particle. Considering this example, the definition and thus the regulation of such a complex concept as "NM" is difficult. *Maynard* also states that "a sensible definition has proven hard, if not impossible, to arrive at" [11, 17].

However, the different definitions are not just pure wordplay. They also have great practical relevance. The fact is that a nanoproduct in the EU does not have to be one in America according to the US EPA definition. An increase of uncertainty among consumers as well as manufacturers is the result. The lack of a general definition of a NM prevents mutual regulation [11]. In the end, the question remains which properties, be it physico-chemical characteristics or even possible interactions between NM and biological systems, are suitable for an adequate definition.

The next paragraphs describe in more detail the novel properties of NMs and their possible applications.

1.2. Physicochemical properties of NMs

The fact that size has a significant influence on the physical and chemical properties of an object and that the subsequent reduction in size leads to new object properties illustrates the potential of NMs (see Figure 1).

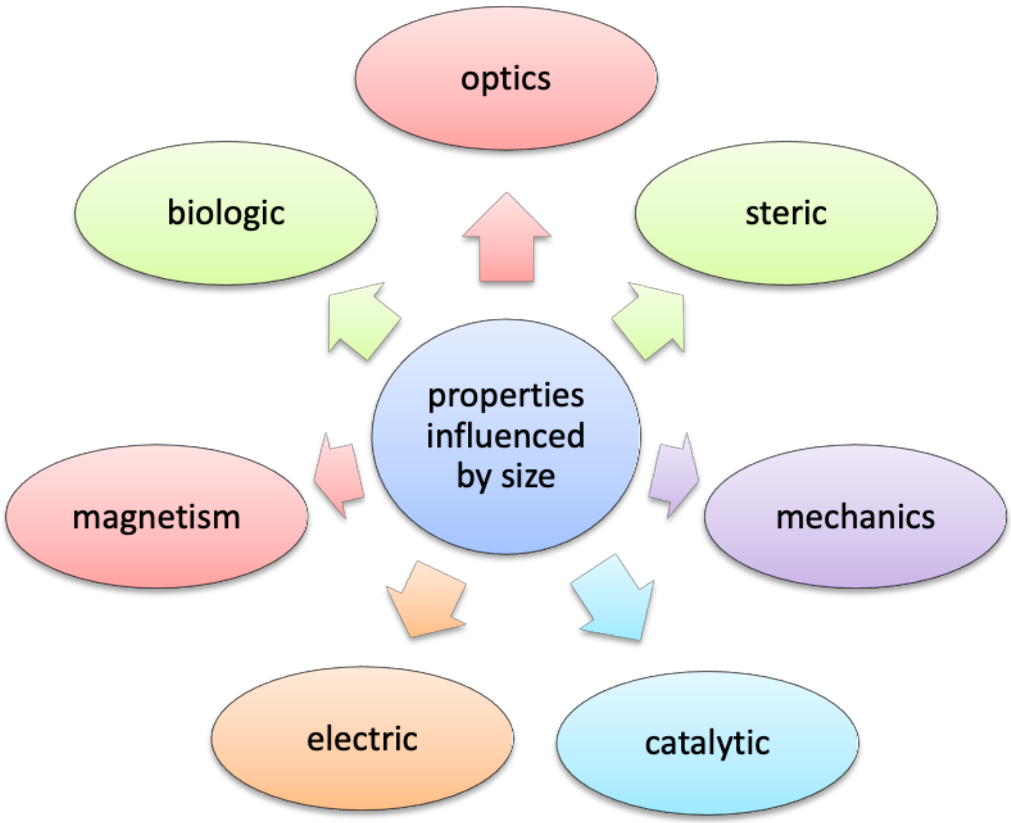

Figure 1 Overview of the material properties that differ from bulk to the nano form (modified from [21])

The following subsections will explain some of the unique properties of NMs and their applications in more detail.

Magnetic NPs can be affected by magnetic fields. The excitation and oscillation of electrons by the magnetic field of light is described as surface plasmon resonance (SPR) [22]. SRP is common for metallic nanostructures and is hardly achievable in other physical states [23]. The application of SPR enables new fields of use for metallic NPs, were the amplification, concentration and manipulation of light are crucial [22, 24] as for the detection of cancer [25]. Furthermore SPR

can not only be exploited as a cancer probe but also to actually attack the cancerous tissue [26]. The so called photothermal therapy is also based on SPR were a heating of the tissue originates from the high energy absorption of metallic NPs and a subsequent energy release in form of heat [27, 28].

Furthermore SPR is employed in sensors which are able to detect biological molecules such as amyloid beta [29] or protein-DNA interactions [30]. The label free detection as well as the possibility to track analyte changes in real time increases the demand for SRP based nano sensors [31, 32].

The resulting manipulation of the structure of the particles by an external magnetic force enables a variety of applications in a wide range of disciplines. Especially in biomedical applications tinier particles in the range of 10-20 nm are preferred if interaction with biomolecules is anticipated [21]. The targeted combination of a single magnetic NP with a biomolecule leads to new approaches and innovations in the field of imaging [33], targeted delivery of drugs [34] or the therapy of medical conditions such as cancer [35, 36]. Besides the biomedical applications magnetic NPs were also successfully employed to clean industrial waste waters [37] and remove heavy metals from drinking water[38].

Due to advances in nanotechnology, NMs are becoming more frequently used in the field of chemical catalysis. In this context they are mainly used as a matrix to support catalytic reactions [39]. Due to their large surface area, they are ideal for carrying a large quantity of catalysts molecules on the surface of the nanoparticle [40]. Compared to conventional methods, the accessibility of the catalyst on nano-based systems is significantly higher than in other types of systems [39]. Especially the reusability of the nano based catalytic platforms renders them interesting for green chemistry approaches [41].

The nano form also changes the mechanical properties of a material when compared to its bulk material, which renders them an interesting option for surface modification to enhance the mechanical strength of e.g. concrete or nanocomposites [42]. NPs are also used as an effective polishing tool [43].

As already mentioned, NPs possess a high reactivity. Upon contact with biological fluids, a complex of layer biomolecules, such as proteins, forms around the particle [44]. The biomolecules compete for the binding to the particle und thus form a highly dynamic envelope, which defines the biological identity of the particle and is called the protein corona [45]. The protein corona contains different layer which are called the soft and the hard corona (see Figure 2). The hard corona contains proteins that exhibit a high affinity for the NP and may even bind irreversible to the NP surface, while the soft corona contains proteins that show a rather low affinity for the particle and may exchange over time [46].

The protein corona influences and alters the physicochemical properties of the NM, such as the size or surface charge [47]. In addition to these changes the particle biodistributions as well as particle induced responses in biological systems are altered through the presence of the protein corona [44, 48]. It could be shown that NPs of the same shape and size showed altered uptake and accumulation behavior when different proteins were present in the culture medium [49].

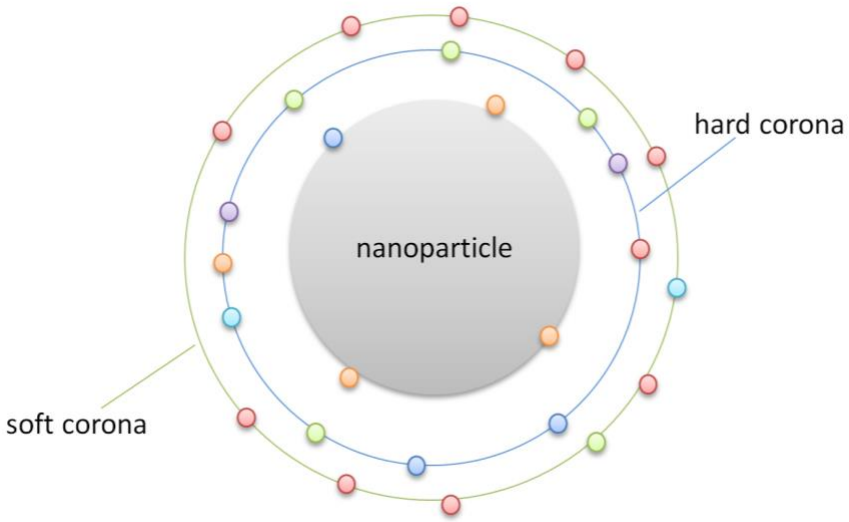

Figure 2 Schematic of NP biomolecule interactions and the formation of a corona around the particle (adapted from [46])

The manipulation of the protein corona in order to increase the efficacy of targeted therapies was already employed for cancer medications. Here, the particle corona was altered with proteins or peptides which were overexpressed in cancerous tissue in order to enhance the targeting of the anti-cancer medication [50]. Since the protein corona changes the identity of the particle in many

different ways, it has to be considered as an important factor for biodistribution and toxicity assessments.

In order to ultimately assess the suitability of NPs for a specific application in a product, the properties and behavior of the NM must be precisely assessed. For this purpose, particle characterization is an important tool to determine such applicability.

1.3. Characterization Techniques

As stated above surface characteristics and size are often criteria for the definition of a NM. A comprehensive physicochemical characterization of the NM is essential to understand the interactions of NMs and cells [51]. However, the underlying relationships between the properties of a NM and the possibly toxic interaction with organisms is still limited [51]. Therefore, a detailed as possible characterization of the physicochemical properties of the NM is recommended [52, 53]. A comprehensive characterization thus contributes to a better understanding of toxicokinetics and the underlying principles which drive the interaction of a NMs and a biological systems [54]. The following part will contain an overview of the most common characterization techniques for NM and highlight their strengths and weaknesses.

1.3.1. Size Characterization

As already mentioned at the beginning, the particle size of a solid substance is a major element for most definitions of a NM. Therefore, the next section will deal with an excerpt of techniques that are able to characterize the size of NM. However, a distinction must first be made here between the hydrodynamic radius, which is made up of the actual size of the NM, an additional hydration layer and polymers or other stabilizers [55]. While the particle core diameter is the diameter of the pristine material.

1.3.1.1. Dynamic Light Scattering

Dynamic light scattering (DLS) is a technique where a laser beam is directed at a sample dispersion. The particles in the sample then scatter the incoming light [56]. The scattering is caused by a Doppler shift of the incident laser light, which is based on the Brownian motion and can be translated into a z-averaged translational diffusion coefficient [57]. Under the assumption that the NM is shaped as a compact sphere the diffusion coefficient can be transformed into the hydrodynamic diameter using the Stokes-Einstein equation [58].

DLS is a very common method for characterizing the size of NMs. Due to the quick and easy handling of this non-destructive technique, the method is suitable to have a first overview of the particles hydrodynamic diameter size distribution of the dispersion [59]. Since the DLS is based

on an intensity weighted correlation function larger particles will dominate the size values over smaller particles which leads to biased results for multimodal particle size distributions [60]. Although DLS has some limitations it is a very valuable tool for a quick NP characterization.

1.3.1.2. Nanoparticle tracking analysis

Like the DLS, nanoparticle tracking analysis (NTA) is based on a light scattering sizing technique and determines also the hydrodynamic diameter. The sample is imaged over a certain period of time using a laser-based microscopy system which allows the visualization and tracking of the particles. The particle follows the Brownian motion and the particle velocity can be transformed in the hydrodynamic diameter by applying the Stokes Einstein correlation.

While in comparison to DLS, NTA can be time consuming it has a better peak resolution and is well suited to characterize polydisperse samples. Especially the presence of very large aggregates has less impact on the NTA sizing accuracy since it evaluates particle by particle instead of the intensity weighted approach of the DLS [61]. However, since large aggregate move very slowly the accuracy of the NTA is decreased. Furthermore, the viscosity of the surrounding matrix strongly influences the particle movement and therefore is one of the limiting factors for the upper detection limit of the NTA.

1.3.1.3. Single particle inductively coupled plasma mass spectrometry

The previous sub-section described particle sizing techniques which are limited in their applicability. For example, the monitoring of environmental NP burden is often not feasible for light-based techniques. In detail the detection limit of these sizing methods is in the mg/L range [60] while the particulate content in complex samples is often in the sub µg/L range [62]. Furthermore, the matrix of human samples, intended for biomonitoring experiments like blood, urine or saliva is often complex and affects the agglomeration and aggregation behavior of the NMs. Especially the characterization of polydisperse samples with light scattering techniques such as DLS is prone to these errors [63]. Single particle inductively coupled plasma mass spectrometry (sp-ICP-MS) can tackle the before mentioned issues.

The sp-ICP-MS is based on a conventional ICP-MS system. The nanoparticulate sample is strongly diluted to a one by one particle sample introduction rate [64]. The liquid sample is then

transported over an inlet into the pneumatic nebulizer. The Nebulizer converts the liquid into an aerosol which is further transported into the plasma where the sample gets ionized. Afterwards the analyte, that has been focused beforehand, enters the actual mass spectrometer. The ionized analyte is separated and detected according to its mass to charge ratio (m/z) [64, 65]. In a typical ICP-MS system, a quadrupole is mainly used for this purpose, although time of flight (ToF) or sector field systems also exist. In contrast to the conventional measurement mode of ICP, where elements can be determined specifically with a high sensitivity, the sp-ICP-MS requires intensity measurements with a very short dwell time (<15 ms). These measurements are performed as a function of time, allowing the identification of above background flashes as individual particles [66]. The frequency of these above background signals is directly proportional to the concentration of particles in the sample [67, 68]. An ionic calibration standard as well as NP reference standard is employed to convert the intensity of the signals into the actual size of the particle [69]. In contrast to other characterization techniques such as DLS or NTA, influences of the matrix such as the formation of a protein corona are not reflected by the particle core diameter which is determined by sp-ICP-MS. This value refers to the actual size of a "pristine" particle. Within the short measuring time of a few minutes, ICP-MS allows not only the determination of the average particle size but also the particle size distribution and particle number concentration of the NPs [64]. In summary, sp-ICP-MS is a powerful tool for the analysis of NPs; however as in any technique there is still room for improvement for example faster scanning rates for a reliable analyte quantification.

1.3.1.4. Microscopy based techniques to analyze size

Currently a number of microscopy based techniques exist that are capable of analyzing NPs. They are not only able to generate images of NPs, but can also provide further information about the elemental composition and structure [60]. All microscopy methods derive their data about the particle properties from the recorded images. Through a suitable thresholding the resulting information is not affected by interference with other particles or the surrounding environment [70]. Meaningful information about the particles within a sample can only be obtained if a sufficient number of particles have been evaluated.

Electron microscopy techniques are all based on the utilization of a high energy electron beam which is directed at the sample. Scanning electron microscopy (SEM) is a technique where the

secondary electrons are measured, which are created while the electron beam scans over the sample. Sample preparation for SEM is lengthy since the sample must be coated with either Au or graphite under vacuum to prevent the interactions of the secondary electrons with the gas molecules. SEM is capable of generating 3D datasets of the sample [60]. The high spatial resolution of SEM as well as the possibility to characterize NMs in all three dimensions exhibits the importance of SEM. Agglomerate formation can hinder the detection of single NPs within these agglomerates [58].

Another electron microscopy based technique is the transmission electron microscopy (TEM). In contrast to SEM the electrons that traveled through the specimen are recorded in order to obtain an image. The sample preparation for TEM is challenging since the sample has to be ideally less than 100 nm thick, to ensure the passage of the electron beam through the sample. However, TEM is one of the most common technologies when it comes to the characterization of NMs and is regarded as the "gold standard" for the size determination of nanomaterials [71].

1.3.2. Composition Characterization

NPs are either consisting of a single constituent or are composite of several materials [72]. Apart from the size of a NM the actual chemical composition plays also a crucial role for the toxicity and the mechanisms involved in the toxicological phenomena observed [73]. For example cadmium containing quantum dots exhibit a strong cytotoxic behavior which is not the case for the non-cadmium containing counterparts [74]. Also the biodistribution within living organism is strongly influenced by the composition of the NM [75]. These examples highlight the need for a thorough composition analysis in order to foster a safe use of NMs.

Standard ICP-MS has become one of the most used technique for quantification and chemical characterization of inorganic NMs [76]. It was demonstrated, that a prior acidification or a microwave digestion of the sample needs to be performed in order to achieve reliable results for the assessment of the NP composition [76].

Another technique to investigate the sample composition is electron energy loss spectrum (EELS) imaging using an electron microscope. EELS is capable of providing information not only on spatial orientation but also on the chemical composition of particles [77]. Like all electron

microscopy techniques, EELS is based on the interaction of a focused electron beam with the sample. During the interaction of the beam with the sample the electrons are scattered. The electrons loose energy in the scattering process. Due to the lost energy of the electrons, the specific composition of the sample can then be deduced [78]. EELS are especially useful for the elucidation of the spatial distribution of the elements.

Instead of an electron beam like in EELS, an X-ray beam can also be used to characterize the composition of the NM. With the so-called X-ray energy dispersive spectroscopy (EDX), a lower background signal can be generated, which leads to an improved quantification compared to EELS [71]. EDX is only suitable for thin samples but is able to detect heavy elements better. EELS, on the other hand, should be used for the detection of lighter elements [79]. In combination with TEM/SEM these two methodologies are powerful tools to analyze and characterize NMs.

1.3.3. Surface Characterization

The AFM is one of the most prominent techniques for surface characterization of NMs and it allows also the determination of the size and shape of an NM [80]. For this technique, the tip of a measuring probe is directed over the surface of the sample. During the process, the tip of the probe interacts with the sample, which changes the oscillation of the probe. The surface topography is mapped using the forces of attraction and repulsion of atoms on the surface and the probe. These subtle changes are detected by a laser directed at the tip of the probe and a photodiode array detector that records the reflections of the laser. An advantage of this method is the rather simple sample preparation. NPs can easily be measured directly on a flat substrate surface. Particle dispersions have to be applied to a wafer beforehand [81].

X-ray diffraction (XRD) can be used to investigate the structural characteristics of crystalline samples. An X-ray beam is directed onto the sample and the resulting diffraction pattern, which is specific for the crystalline structure of the sample, is assessed [81]. When evaluating XRD experiments, however, it should always be remembered that the information obtained is derived from the crystallographic properties of the corresponding crystal domains [82]. Unfortunately, these domain properties do not always correspond to the properties of the whole particle. It is

therefore necessary to use additional characterization techniques like TEM to verify the results form XRD experiments [81]. Nevertheless, XRD is a valuable tool not only for the structural characterization but also for the determination of surface charge, shape and area of a NM.

A technique which can be employed for the determination of the three dimensional structure as well as for the determination of the surface and chemical composition is the time of flight secondary ion mass spectrometry (ToF-SIMS). This method relies on the detection of secondary ions which are formed after the sample is impacted by a primary ion beam. This technology is especially well suited for the detailed three dimensional visualization of multiple chemicals, metabolites within biological samples and also for the characterization of pristine NMs and NMs within biological samples [83, 84]. The high lateral resolution down to the sub-micron range in combination with the extreme depth perception of this technique allows the visualization of cells as well as NMs in a high level of detail [84]. However, the long duration of the analysis and the rather complex sample preparation are possible shortcomings of this technology.

1.3.4. Benefits of a comprehensive characterization

The methods presented above can record the properties of NMs in detail. The further development of new NMs with specific characteristics as well as the reliable manufacturing of NMs requires inexpensive and reliable characterization methods. The advancement of existing techniques is therefore equally important as the emergence of new characterization techniques [81].

Due to the large number of modifications of NMs, comprehensive toxicity testing of all NMs is neither financially nor time-wise possible. In order to nevertheless make statements about the toxicity of a NM, they are grouped according to their physical and chemical properties. The biological interactions are then tested for only a few representatives of each group and the results are applied to the whole group [85]. However, since changing the properties of a NM alone can lead to altered biological interactions, grouping is a challenging process [86]. In addition, the fact that in most of the characterization studies the NM was examined individually is a complicating factor. In biological environments NMs are modified for example by the formation of a protein corona and thus their reactivity in biological systems may change accordingly [87]. Nevertheless

grouping is considered to be a valuable approach to fill data gaps, reduce animal experiments and provide a valid prediction tool for the safety assessment of NMs [86].

A comprehensive physicochemical characterization does not only serve the purpose of obtaining information about a NM. Since properties of materials also steer their interactions with the environment, this knowledge can be used to predict the interactions between NM and the environment, uptake into organisms and resulting toxicity [86].

1.4. Biokinetic behavior of particles

The versatile use of NMs opens up numerous possibilities of exposure scenarios for humans. Although a multitude of products contain NMs the actual knowledge about the release of NMs from a product is still very limited [88]. For the majority of people, air, food and consumer products which are contaminated with NPs represent the greatest risk of exposure. For humans the three major uptake routes are uptake via the dermal, respiratory and oral barrier [89]. The following section will deal in more detail with possible admission mechanisms into the body and the subsequent cellular uptake mechanisms.

1.4.1. The dermal barrier

Every substance which comes into to contact with the skin might be able to cross the dermal barrier, get absorbed into the skin tissue and may get systemically available. The use of NMs in cosmetics suggests that there might be a risk of dermal absorption.

There are essentially two routes for uptake into the skin: through the skin appendages or through the stratum corneum. Studies which employed fluorescent polystyrene NPs were able to show that NPs tend to accumulate in follicular openings of porcine skin in a vertical diffusion cell. However, no further evidence of particle absorption was observed [90]. Although particles can accumulate in hair follicles this process is by definition not an uptake into the body [91]. Furthermore, the lower end of the hair follicles is protected by a layer of tight junctions [92] which remain intact upon exposure to particles. An uptake of particles through follicular penetration was not observed for insoluble NPs [93].

The skin consists of several layers of different cell types which form a robust barrier. The Epidermis, which is the outermost part of the skin, is renewed every 4-6 weeks [94]. The continuous renewal of the epidermis can be a decisive factor in preventing the systemic availability of NMs [95]. The stratum corneum (SC), which forms the outermost layer of the epidermis, is for many substances the decisive barrier for absorption through the skin [91]. The brick and mortar structure of the SC offers a substance the possibility to either pass through the corneocytes or penetrate along the lipid matrix. The uptake by following the lipid channels

between the corneocytes is more likely to occur since the lipid matrix offers a channel like structure that facilitates the diffusion [91].

Skin penetration studies which utilized quantum dots showed increased skin penetration [91] while for insoluble NPs such data is quite rare. An uptake into the skin has been demonstrated for titanium oxide particles [96]. Scientific evidence for a systemic availability of NMs after contact with intact skin remains scarce [97]. However, a case report of a burn victim which was treated with a special burn dressing that contained silver (Ag) NMs showed elevated Ag blood plasma levels in the patient [98]. Studies suggest that already a mild damage like a sunburn is sufficient to increase the uptake of NMs which are present in skin care products [99]. These findings demonstrate that an impairment of the skin barrier increases the likelihood of an uptake of a NM into the skin.

In summary, the available scientific data suggests a rather low uptake of nanomaterials into the intact skin. Nevertheless, a better mechanistical understanding of the cellular uptake mechanisms of NMs is necessary in order to achieve a reliable risk assessment.

1.4.2. The inhalative uptake

While the skin is often regarded as less penetrable, the respiratory route is regarded as a more relevant entry point of NPs into the body. NPs that are present in the airflow can reach the lung tissue during breathing and are further translocated [100]. The understanding of the underlying deposition mechanisms and factors which influence the deposition are crucial for a reliable risk assessment [101] The deposition of airborne particles depends on multiple factors, whereby the major ones in this context are the properties of the aerosol itself, the particle diameter and the inspiratory flow rate [101]. Larger particles and agglomerates, which have settled in the area of the upper respiratory tract, are contained in a mucus layer. Through mucociliary clearance these particles are now transported to the throat where they can either be coughed out or swallowed [102].

Particles which are smaller than 500 nm are able to travel from the extrathoracic parts of the respiratory tract all the way down to the alveoli mainly through diffusion [103, 104]. The alveolar region consists of alveolar epithelium which is formed by Type I and II pneumocytes as well as a

one cell layer thick endothelium and an interstitial cell layer. The small distance between the capillaries and the alveolus allows the exchange of gas which is driven by diffusion. A liquid film which is called surfactant and consists of phospholipids and surface proteins covers the alveoli. It enables an effective gas exchange by lowering the surface tension [105]. The surrounding tissue of the alveoli contains fibroblasts and macrophages. The later ones help to prevent pathogens from infiltrating the organism and can trigger a rapid immune response [106].

The particles which reached the alveolar region are first confronted with the protein containing surfactant. Since NPs have the unique feature of forming a protein corona, they are able to attach proteins from the surfactant onto their surface. This can result in a disturbance of the particle clearance as well as a decrease of the surfactant production [107].

Particles that have been submersed in surfactant can be taken up by lung associated macrophages. These macrophages play a crucial role in the elimination of the particles but are also contributing towards particle retention in the body [103]. Experiments in rats demonstrated the ability of macrophages to take up 90% of the remaining lung retained 20 nm iridium NPs in a time course of 72 hours [108]. After the uptake the particles are transport to the larynx, which is considered the major clearance mechanism for the peripheral lung regions [103]. However also the characteristics of the particle seem to play an important role for the macrophage mediated clearance. A study which employed 20 nm TiO_2 NPs showed a rather low particle uptake of 0.1 % within the first 24 hours of inhalation [109]. For microparticles of different origins the phagocytose rate after 24 hours is expected to be 85-90% [103]. This highlights that the particle size and chemical composition seem to play an important role for the clearance mechanism. Furthermore the data suggest, that the main clearance mechanism is much slower for nano sized particles and thus increases the probability of uptake into the epithelial cell layer or even across the epithelial barrier [103]. Experiments suggest a crossing of the epithelial barrier and a further translocation of the particles in secondary organs. This was shown for metal as well as for non-metal containing particles in the size range between 5-100 nm in animal experiments [100, 103].

Experiments in which healthy adults inhaled Au NPs were able to reproduce the results of the animal experiments [110]. *Miller et al.* could demonstrate a prolonged Au excretion even 3 months after the exposure with the 5 nm Au particles. These findings suggest a systemic retention

and delayed excretion over the urine for the 5 nm Au NPs [111]. They furthermore could show a translocation of the systemically available Au NPs in atherosclerotic lesions in the human test subjects who had a history of ischemic stroke [111]. In summary, the results of Miller's study highlight an uptake of airborne NPs and their deposition in dangerous regions of the body such as atherosclerotic altered blood vessels. In particular, the apparently favored deposition in inflamed tissue should be a cause for concern since the particle mediated toxicity could worsen the state of the inflammation and potentially harm the organism. Unintended exposure of humans to NPs should be prevented when possible [111].

As in the case of initial particle deposition in the lung, several properties of the particles play a decisive role in their uptake and further translocation into the body. It was shown that a large surface area, which is increasing with decreasing particle diameter as well as a negative particle charge are key parameters for an enhanced translocation [112]. The solubility of a NM dictates its retention time in the lung tissue and the subsequent clearance mechanism [100]. As already described earlier the protein corona of the particle also influences the particle translocation and the further uptake and translocation route throughout the body [113].

The results of numerous studies show that although microparticles and larger aggregates of particles are efficiently removed from the lung due to mucocilliary clearance, NPs can circumvent this mechanism and are able to translocate into the body and have the potential to cause toxicological relevant adverse health effects.

1.4.3. The oral uptake route

Since NPs are used as food additives and in products that are in contact with food, oral intake is a plausible uptake route in addition to inhalation [114]. The particles are either intentionally added to the food product or might migrate into the food from packaging materials or during the cooking process and are then ingested. While it was demonstrated that a release of NMs from low-density polyethylene (LDPE) based food contact materials is rather unlikely [115, 116] the NM release from other nanocomposites might occur and has to be taken into account [54]. During the digestion, the particle travels through the compartments of the gastrointestinal tract [117]. In each of these compartments the environment of the NP changes drastically. Especially the pH shifts as well as the presence of different enzymes changes significantly from

compartment to compartment. Salts and bile acids may as well influence the properties of particles [118]. A change in the particle properties might result in a changed agglomeration behavior or even the complete dissolution of the NPs [117]. The enhanced dissolution of the particle and the subsequent formation of ions naturally pose far-reaching problems for risk assessment because the absorption of metal ions from the intestine will show significantly different kinetics than the uptake of particulate components. In order to elucidate the changes of the NP properties during the digestion process an artificial digestion procedure was used by *Sieg et al.* [117]. Their results clearly indicate a *de novo* formation of particles which originate from free ions in the nano-range as well as a dissolution of the administered particles [117]. These results highlight the importance of particle characterization under physiological conditions in order to predict the exact uptake of a NP into the body.

Particle absorption is a prerequisite for the particle translocation into secondary organs. The decisive factor for the adsorption is the passage of the particles or ions through the intestinal epithelium [119]. However, before particles can enter the epithelium, they have to pass through the mucosa of the gastrointestinal tract. The mucosa is a gel-like fluid that covers the surface of the gastrointestinal tract and is therefore a barrier for both macro- and NPs. As the mucosa is continuously reproduced, particles contained in the mucosa are transported along the gastrointestinal tract and excreted via the feces [120]. Particles which were able to defy the mucosa are mainly absorbed by the M-cells which are in the Payers patches of the small intestine and also by the enterocytes of the intestine [121]. A study with non-degradable latex particles demonstrated that with decreasing particle diameter increases the probability of absorption into the cells increases. The majority of taken up particles in this study were in the size range between 50 and 100 nm [122]. The uptake rate of particles can be further enhanced by an optimized composition, or by the application of ligands on the particle surface which can interact with receptors responsible for the uptake of substances into the cell [123]. After crossing the intestinal barrier, the particles are either transported into secondary organs by the bloodstream or via the lymph [124].

1.4.4. Distribution of bioavailable NPs

From the previous sections of this chapter it is evident that NPs are capable of overcoming the gastrointestinal as well as the lung barrier and thus become systematically available. Only the intact skin seems to be a rather insurmountable barrier for NPs. The following section describes the distribution and accumulation of NPs in the body.

After the NPs enter the blood stream they are in contact with the constituents of the blood [125]. Since NPs can form a protein corona, this protein corona may now change depending on the interactions with various blood constituents. The resulting formation of the protein corona will significantly increase the NPs size compared to the pristine particle [126]. The particle will then be transported through the body by the blood circulation. Its half-life time in the circulation depends strongly on its size. Particles smaller than 5 nm can overcome the endothelial barrier much faster than larger particles and show a prolonged circulation half-life time compared to larger particles [126]. Besides size, surface modification plays a major role in the biokinetics of the particles. It has been shown that pegylation significantly alters the half-life in blood and influences the distribution patterns in secondary organs significantly [127]. By specific surface modification it is therefore possible to vary parameters which are particularly important for the therapeutic application of NPs [127]. Another parameter which seems to contribute to the half-life time in blood is the surface charge. *Arvizo et al.* were able to demonstrate that negatively or positively charged NPs exhibited shorter circulation half life time compared to neutral ones [128].

NPs are hence capable of leaving the blood stream over time. Animal studies have shown that the majority of particles are found in the liver and spleen [100]. However, more specific information about the target organs and tissue distributions of metallic NPs is scarce and a clear need for research exists [129]. Moreover, there are also differences within the group of metallic NPs, which further complicates the problem. For example, the biotransformation of metallic particles that easily dissolve and form ions such as Ag differs from that of those that are rather insoluble like Au based NPs [129].

1.4.5. Cellular uptake mechanisms of NPs

After a NM has left the bloodstream and migrated into a tissue, it can be absorbed into the cell. In general, the uptake of NMs follows a common pattern. The material reaches the outer cell membrane, interacts with it and is absorbed by an endocytosis mechanism [130]. Endocytosis is

an uptake process in which the cell membrane encases the object, resulting in the incorporation and formation of an endocytotic vesicle. A distinction is made between different endocytosis pathways which can be differentiated according to cell type and involved components [131].

1.4.5.1. Phagocytosis

Phagocytosis is primarily but not exclusively performed by macrophages, dendritic cells, monocytes and neutrophils as part of the immune response and of the removal of cell debris [130]. Opsonization, a process of tagging an object by immunoglobulins, components of the blood or complement system is required before phagocytosis can occur [132]. This tagging process enables the object identification via receptor ligand binding and subsequently it is incorporate into the phagosome [133]. Similar to all previously mentioned interactions of NMs and organisms, the physico-chemical properties play a key role in the whole uptake process. It has been shown that agglomerates and larger particles in the range between 1000 and 2000 nm are preferentially taken up by macrophages by means of phagocytosis [134]. Particles that can form a protein corona due to their charged or hydrophobic surface are more likely to be opsonised than particles that do not have this features [133]. Opsonisation can be prevented by PEGylation and other surface modifications, which prevent the interaction of biomolecules with the surface of particles [135].

1.4.5.2. Clathrin-mediated endocytosis

Cells depend on the absorption of nutrients and lipoproteins from their environment to maintain their homeostasis. Clathrin-mediated endocytosis (CME) is the regarded as the main uptake pathway for those substances [136]. This type of absorption takes place at Clathrin-rich regions of the plasma membrane. The uptake mechanism can either be receptor mediated or receptor independent. After the particle agglomerate has reached the cell, Clathrin and other accessory proteins form an invagination in the plasma membrane and facilitate the uptake of the cargo into the cell [130]. Investigations have shown that particle agglomerates taken up via this route often end up in degrading lysosomes [137]. Both surface modification and charge influence the preferential uptake by the cell. It could be shown that both poly(ethylene glycol-co-lactide) [138] and D,L-polylactide (PLA) [139] modified NPs are absorbed into the cell via CME. If the CME uptake is blocked by inhibitors, the uptake into the cell was significantly reduced in cell culture

systems. The charge of the particle not only influences the preferential uptake route but also their further intracellular metabolization [130]. Positively charged NPs are strictly taken up by CME in HeLa cells while anionic particles demonstrated an uptake over either CME or caveolae-dependent endocytosis [138].

1.4.5.3. Caveolae-dependent endocytosis

Caveolae-dependent endocytosis (CDE) is another important uptake pathway for NPs. From a physiological point of view, CDE is relevant for the regulation of lipids and membrane components as well as for signal transduction [130]. Caveolae are membrane areas with a size of about 50-80 nm which are lined with caveolin. After binding of the particle agglomerate, the membrane is inverted and a vesicle containing the absorbed cargo is constricted [140]. Subsequent lysosomal degradation can be bypassed by some particles, rendering CDE uptake a preferential target for gene or protein based therapies [141].

1.4.6. Exocytosis of NPs

While the different endocytosis mechanisms and ways to exploit them for therapeutic options are in the focus of research, the exocytosis of particles remains largely unexplored. NPs located in a lysosome can be transported via an active transport into the periphery of the cell and either taken up by another lysosome or released from the cell by exocytosis [142]. It has been shown that particles leaving the endocytic vesicle or the lysosome and are thus present in the cytoplasm are excreted significantly slower than particles in such a vesicle [143]. The exocytosis process itself relies on cholesterol and sphingolipid rich membrane domains as excretion sites [144]. After leaving the cell these particles can than enter other compartments of the body for example the brain by penetration of the blood brain barrier and may cause toxicity [142, 145]. It was demonstrated that exocytosis rates of particles with the same physicochemical properties differ from cell line to cell line [142].

1.4.7. Clearance of NPs contained in the blood stream

A major part of the particles that reach the blood stream is excreted in the course of time, provided the particle has not left the blood stream. Renal clearance followed by urinary excretion

is one of the major excretion pathways for particles that entered the bloodstream [126]. As already described for the uptake of the particles, the physicochemical properties are also decisive for the elimination. A study that used quantum dots to investigate renal clearance has shown that renal clearance is possible for particles with a hydrodynamic radius smaller than 8nm [146]. These results are consistent with data on renal protein clearance, therefore there seems to be no particular nanospecific clearance mechanism [126]. Apart from the primary particle size, the charge of the particle plays a crucial role. Anionic or cationic particles have demonstrated the ability to adsorb serum proteins and form a distinct protein corona. The adsorption leads to a substantial increase of the hydrodynamic radius and thus may reduce renal clearance [146]. Once the particles have been successfully excreted into the primary urine, resorption of the particle in the proximal tubule may occur. Unfortunately, there is no reliable data on the behavior of particles in the proximal tubule. Since the kidney and especially the tubule epithelium is a preferred target for heavy metals, there is an urgent need for further research [126].

NPs that have reached the blood stream can also be removed from systematic circulation through the liver. Due to the high blood perfusion and fenestrations in the endothelial cells of the liver, NPs tend to passively accumulate in liver tissue [147]. In the tissue of the liver, the particles encounter hepatocytes or Kupffer cells. Kupffer cells are differentiated macrophages of the liver, which can phagocytize and degrade foreign matter such as particles [148]. However, particles that cannot be degraded by macrophages remain in the cell and thus also in the body. Any particles that have been taken by hepatocytes through endocytosis can be degraded enzymatically and then excreted through the bile [126].

1.5. Toxicity of NPs

It is assumed that the physico-chemical properties determine the interactions of the particles with their environment and thus also define the toxicity of the particles [149]. In the beginning of this thesis, the different physico-chemical parameters of NPs were described in detail as well as methods for the appropriate investigation. In the following section the different modes of particle induced toxicity will be investigated further.

The size of the particles is obviously a crucial parameter for particle toxicity. As mentioned in the previous chapters, size is a decisive factor for the biokinetics of the particle. The particle surface area increases with decreasing size. Due to the increase in surface area, increased interactions of the particle surface with the surrounding environment of the particle are possible [150]. It has indeed been proven that the surface area of a NP is most predictive for particle associated acute toxicity in lung tissue [151]. The surface charge of a NM determines not only the formation of the protein corona and the subsequent biodistribution of the particle agglomerate but also the toxicity of a particle. In general a positive charged particle tends to exhibit a stronger toxic behavior [152]. Whether this behavior is due to an enhanced uptake or an altered biodistribution profile remains unclear. Lastly the shape of the particle also plays a decisive role for the overall toxicity of a NM, as the example of carbon nanotubes illustrates. Similar to asbestos, increased inhalation of the fiber like carbon nanotubes leads to chronic inflammation of the lung tissue [153]. In addition to the numerous *in vivo* studies which were able to explore the relationship between the inherent particle properties and the caused toxicity, *in vitro* studies were also conducted to elucidate the underlying toxicity mechanisms in cellular compartments.

One of the best studied NP related toxicity mechanisms is the generation of reactive oxygen species (ROS) by metallic NPs [149]. A direct correlation between the inhalation exposure to metallic NPs, the resulting production of ROS and proinflammatory reactions could be demonstrated [154]. Under physiological conditions, small amounts of ROS are produced in cells. For example, ROS are formed as by-products in the mitochondria during the energy metabolism. These ROS can inflict damage to lipids, proteins and nucleic acids [149]. They are therefore not only able to severely damage cellular structures such as mitochondria but also to

disrupt the functionality of proteins [155] With the help of antioxidants such as glutathione (GSH) system and antioxidant enzymes, these physiological occurring small quantities of ROS can be quickly and effectively eliminated before damage to cellular structures is dealt [154, 156]. However, exposure to NPs and the resulting ROS cascade can deplete the antioxidative capacity of the cell. Consequently, GSH is completely diminished and the concentration of the oxidized form of glutathione is rising. This circumstance is also known as oxidative stress [154]. Cells which are exposed of oxidative stress for a prolonged period of time often die to the increasing damages of the ROS. Cell membranes become damaged and the membrane integrity is disrupted which results in a loss homeostasis [157]. DNA damages occur in higher frequencies which exceeds the capacity of the DNA repair machinery [158] and leads to cell cycle arrests and the induction of apoptosis [149].

Furthermore, NPs are able to reduce the membrane potential of mitochondria. This damage massively disrupts the energy production of the cell and it is very likely that these cells will enter apoptosis. This mechanism has been successfully demonstrated for various cells and NPs [149].

The interaction of NPs and proteins may change the protein conformation and hamper their functionality [159]. The unfolding of the protein structure might also cause accessibility of beforehand hidden protein motives. Those newly exposed motives can trigger a broad range of unsolicited cellular replies. For example Au NPs were capable of binding and unfolding fibrinogen which in turn activated THP-1 cells that released a proinflammatory response [160]. Other studies showed that NPs were capable of inducing fibril formation [161] which is a key event in the development of neurodegenerative diseases such as Alzheimer or Parkinson disease [159]. In addition to the before mentioned mechanisms, metalloproteins are a target for metallic NPs. The metallic ions interact with the metals bound within the protein and are able to severely hamper the functionality of the metalloprotein [162].

The toxicity mechanisms mentioned above are able to cause enormous damage in the cell. However, exposure to NPs does not always lead to the induction of apoptosis or even necrosis [163]. It is also possible that the inflicted damage to proteins, DNA and cell membranes leads to a cell cycle arrest [149]. Minor damages may be fixed with the help of cellular repair mechanisms while irreparable cell damages will lead to the induction of apoptosis or even necrosis [164].

Cells which are contained in cell cycle arrest do not undergo proliferation. The suppression of cellular proliferation is another mode of NP toxicity [149].

The above mentioned toxicity mechanisms can be influenced by the physico-chemical properties of the NMs. The complete elucidation of these processes will enable the development of safe nanoproducts. However, there is still much research needed to understand the underlying relationships between NPs and biological systems [165].

Comprehensive material characterization is the first step in understanding the interactions between NMs and the biological system. There is still a great need for knowledge in order to determine precisely which material properties lead to interactions and their resulting toxicity mechanisms.

2. Aim of this thesis

Nanotechnology offers the possibility to provide novel products and innovative solutions. The areas of application of nanotechnology are diverse and cover all aspects of life. In my opinion, this technology has the potential to solve existing problems, be it the improvement of energy storage, the advancement of catalysis reactions or the targeted application of pharmaceutical agents. However, all these solutions are accompanied by new hazards regarding the safe use of these products. The overarching aim of this thesis is to contribute towards the understanding in which NP interactions can be studied and thus ensure a safe and effective use of this technology.

As already explained in the introduction, the definition of a NM refers primarily to one parameter: Size. While size definitely contributes to several biokinetic properties, there is a variety of techniques available to determine the actual particle size. A further development of existing techniques can contribute to an improvement in the accuracy of particle characterization. Within the scope of this thesis a sp-ICP-MS based size distribution characterization approach was implemented in order to expand and improve the available methodology for NP characterization (chapter 3.1).

NMs are widely used in food and food contact materials. Further research is required to determine the risk of such substances, especially regarding the uptake and the distribution within the body of a NM. Therefore a 3-day oral uptake study was conducted with a special focus on the secondary organ distribution and the difference in bioavailability of the two administered aluminum species (chapter 3.2).

In addition to the oral uptake *in vivo* experiments, it is essential to gain deeper insights into the underlying interactions of particles with cells and cell organelles. In the chosen experimental setup human keratinocytes were exposed to the same two aluminum NPs that were already used in the conducted 3-day oral up take study. Further insights into NP distribution and membrane changes induced upon exposure in combination with the application of bioactive substances were the goal of the study. The results improve the understanding of adverse effects that particles induce and they might also lead to the development of safer nano-based products (chapter 3.3).

The enhancement in the three major pillars for NM risk assessment: physicochemical characterization as well as toxicological data from *in vitro* experiments and lastly also from *in vivo* experiments will contribute to a holistic health based risk assessment approach for nanomaterials.

3. Results

The publications shown here are not in chronological order due to a better comprehension of the thesis. Abbreviations and units have been defined within the respective publication. Furthermore, each publication states the respective author contribution.

3.1. Improved validation for single particle ICP-MS analysis using a pneumatic nebulizer / microdroplet generator sample introduction system for multi-mode NP determination

Daniel Rosenkranz[*,§], Fabian L. Kriegel[*], Emmanouil Mavrakis, Spiros A. Pergantis, Philipp Reichardt, JuttaTentschert, Norbert Jakubowski, Peter Laux, Ulrich Panne, Andreas Luch

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The supplementary material of the publication can be found in Annex I.

3.2. Aluminum and aluminum oxide NMs uptake after oral exposure - a comparative study

Benjamin C. Krause[*§], Fabian L. Kriegel[*], Daniel Rosenkranz[*], Nadine Dreiack, Jutta Tentschert, Harald Jungnickel, Pegah Jalili, Valerie Fessard, Peter Laux & Andreas Luch

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The supplementary material of the publication can be found in Annex II.

3.3. The Vitamin A and D Exposure of Cells Affects the Intracellular Uptake of Aluminum NMs and Its Agglomeration Behavior: A Chemo-Analytic Investigation

Fabian L. Kriegel[§], Benjamin-Christoph Krause, Philipp Reichardt, Ajay Vikram Singh, Jutta Tentschert, Peter Laux, Harald Jungnickel and Andreas Luch

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The supplementary material of the publication can be found in Annex III.

4. Discussion

The following section comprises a detailed result discussion of the presented work.

4.1. Enhancement of the sp-ICP-MS methodology

Size characterization of NPs using sp-ICP-MS is one of the most frequently used applications of ICP-MS. For an effective ionization of the sample by the plasma, a suitable sample introduction system must be used. In order to achieve optimized ionization for single particle ICP-MS a pneumatic nebulizer (PN) is applied in most of state-of-the art ICP-MS instruments, but it must be noted that only a fraction of the sample reaches the plasma after passing through the PN. To adequately account for this loss, the PN based system must be calibrated using a certified reference size standard in order to assess the so-called transport efficiency. The use of such a certified reference standard has certain disadvantages. For example, the number of available size standards for NPs is very limited, which leads to the use of standards that do not match the ionization capability of the particle to be analyzed. In addition, PN based systems also have multi-particle events per dwell time, which further increases the measurement uncertainty of this methodology. In the presented publication, the use of a size reference standard was circumvented by using a microdroplet generator (MDG) as sample introduction system. However, MDGs are not without any disadvantages either, the system must be elaborately aligned, and the necessary parts must be adapted to the respective device in a laborious process. A dual inlet system consisting of PN and MDG have already been presented in previous work by *Ramkorun-Schmidt et al.* with the aim of exploiting the advantages of both sample introduction systems [166]. However, the construction they presented had a considerable room for improvement.

First, we succeeded in designing a dual interface which can be implemented using standard parts normally available in a routine ICP-MS laboratory [65]. Therefore, our approach saves additional costs and is easily adaptable to every ICP-MS device. The design of *Ramkorun-Schmidt et al.* enabled them to combine both sample introduction systems, however, lengthy washing steps between samples had to be performed to avoid clogging or contaminations. By using flexible tubings and suitable carrier gas flows we were able to improve these deficiencies significantly

and therefore guarantee a continuous sample introduction flow over time, which significantly reduced overall analysis times for each experiment. In contrast to other approaches, the system developed by us does not have to be disconnected from the ICP-MS system in between samples. It can therefore be cleaned by applying rinsing solutions without shutting down the ICP-MS device. Compared to similar systems our system has a great potential to save costly analysis time, since shutting down and restarting an ICP-MS is a time-consuming process and would also require additional tuning steps to guarantee comparability between the measurements.

Although *Ramkorun-Schmidt et al.* demonstrated the accurate determination of particle sizes as well as metal mass fraction using the dual inlet system, they utilized just one way to analyze their data. We have approached this issue and implemented further data analysis modes in our study, which are based on the use of the respective sample introduction systems. Finally, we succeeded in creating an ICP-MS based measurement procedure where a NP dispersion can be characterized within a single 20 min run by using three different data analysis modes. Within the scope of our study, we used the developed setup to characterize three different NPs: Au 56nm, Ag 75 nm and the polydisperse CeO_2 10-100nm. In brief the run consists of 4 consecutive measurements, 2 for the ionic and 2 for the particulate solution. First an ionic solution is measured using the MDG and afterwards the PN. In the next step the actual particle containing solution is introduced into the ICP-MS using the MDG and finally the PN.

For the actual data analysis and interpretation, we compared three different modes. Each mode of analysis is based on a unique combination of operation and calibration procedures, which will be briefly discussed below. Mode I is the widely used approach for sp-ICP-MS characterization of NPs developed by *Degueldre et al.* [67]. In this mode only the PN is used as the sample introduction system for the ionic as well as the particulate solution. Accordingly, a reference standard must be applied in order to determine the transport efficiency of the system. For the NPs of Ag and Au we were able to determine diameter and particle number concentrations, which differed minimal from the manufacturer's specifications. The good agreement for these two particles proves that our developed setup does not affect the measurement process itself. For the polydisperse CeO_2, which has a size distribution between 10 and 100 nm, we obtained a mean particle diameter of 61.9 nm. This deviates significantly from the mean particle diameter of 28.4 nm of the manufacturer which was determined by TEM. As we were able to show such a

deviation in all three modes of analysis, the evidence points to an agglomeration of the sample. However, the determined mean particle diameter is within the defined size range of the CeO_2 dispersion.

In analysis mode II an ionic solution is introduced in the ICP-MS using the MDG as well as the PN and a particulate solution is later measured using the PN. This approach was developed by *Ramkorun-Schmidt et al.* [166]. It utilized the signal which the ionic solution generates while being introduced over the MDG to determine the ICP-MS response factor. This factor is then used to convert the ion intensity per NP into the metal mass per NP. We applied this methodology and obtained similar results as with analysis mode I. The results for Au and Ag NPs are in good agreement with the manufactures data, however for the polydisperse CeO_2 NPs we obtained an even higher mean particle diameter of 70.6 nm. Agglomeration of the particles in the suspension is most likely the reason for our findings. However, the use of analysis mode II also has a decisive disadvantage. In order to determine the transport efficiency of analysis mode II, which is necessary to calculate the mass of the particle and thus its size, the nanoparticulate sample must be digested [166]. This is called the ion intensity approach for transport efficiency determination. Such a digestion procedure is often very labor intensive and time consuming especially for very insoluble metals and metal oxides like titanium.

In order to overcome this obstacle, we utilized the high transport efficiency of the MDG which is ensured in our setup due to the application of a helical makeup gas flow. The MDG derived transport efficiency of approximately 100% allows the determination of the transport efficiency of the PN. The mass sensitivity obtained by the PN divided by the mass sensitivity of the MDG results in the transport efficiency of the PN. In analogy to the use of mass sensitivities, this approach for the determination of the transport efficiency was referred to as the sensitivity approach. The results obtained by the sensitivity approach are alike to those of the other two modes and therefore show the reliability of this methodology. The major advantage of the sensitivity approach is its applicability for any element detectable by ICP-MS with no requirement for a referenced size standard. As a result, the sensitivity approach is very versatile and, since no special standards are required, it should be the first choice for characterizing NPs for which no referenced size standards are available.

For analysis mode III the ionic as well as the particulate sample is introduced over the MDG while the PN transports a constant stream of 0.5% HCl into the ICP-MS. This approach was already described in detail by the group of Günther and colleagues [167]. The transport efficiency in this approach is also known to be 100% since a MDG is used. The results for the investigated Au and Ag NPs are again in agreement with the manufacturer whereas for CeO2 a mean diameter of 74.4 was determined. The results obtained via Mode III are equal to the results of the other two modes.

The aim of the study is certainly not to prove the superiority of one mode of analysis over the other but rather to encourage the use of all three modes and to compensate the respective strengths or weaknesses of the sample introduction systems and thus contribute to an improve characterization of NMs. However, in some cases there may be NMs where high transport efficiency is necessary for their detection due to low sample volumes or concentrations. In addition, a reference material-free characterization of the NM offers an increased reliability of the results.

For a further improvement of the sp-ICP-MS methodology it is essential to account for sample matrix effects in the characterization process. The matrix has a direct influence on the accuracy of the characterization and may therefore lower the accuracy and reliability of the characterization. Therefore, it would be desirable that in the future comprehensive studies are carried out with the objective of finding the most suitable approach for sample introduction and the corresponding mode of analysis. The results of such studies can contribute to a rapid and high throughput analysis of real samples.

It is also worth mentioning that our instrumental equipment only allows for a single-element analysis of the NPs. However, since the use of NPs consisting of several elements is increasing, the application of an ICP- time of flight (ToF)-MS due to higher scan rates and the resulting higher mass accuracy is required for a simultaneous detection of those elements. It is expected that our setup would also be applicable for a multi element analysis on such an ICP-ToF-MS.

The influence of matrix effects does not only have a decisive impact on sp-ICP-MS analytics. Chapter 4.2 will provide an in-depth look at the influence of organic matrices on the detection of aluminum NPs in rats as well as their tissue distribution following oral administration.

4.2. The oral uptake of aluminum NPs

Health concerns arise whenever limit values for hazardous substances are almost exhausted or even exceeded. Dependent on their composition and the degree of exposure, NPs contribute to the total human exposure burden. Increased use of nanotechnology may therefore result in the exceeding of such limit values. The question of whether NPs lead to higher human exposure in comparison to ionic compounds is mostly unresolved. At the moment the nanoform is not taken into account for any threshold values for hazardous substances.

In the study presented in this thesis, aluminum-based NPs (Al^0 20 nm, Al_2O_3 20 nm) and the soluble $AlCl_3$-$6H_2O$ were applied. Aluminum represents a potentially hazardous element which is applied in consumer products, food additives and packaging [168]. Due to its advantageous properties, nanoscaled aluminum applications for consumer products are on the rise. Increasing exposure of consumers to aluminum is a cause for concern, as an association between enhanced aluminum exposure and neurodegenerative diseases such as Alzheimer's have been discussed in the past years.

Reliable information about the biokinetics of nanoscaled aluminum is also still sparse. In order to further promote the scientific discourse, a 3-day oral gavage in study was performed in the frame of the SolNanoTOX project. Aluminum-based NPs were selected for exposure of Sprague Dawley rats. An oral uptake via gavage was chosen as the route of exposure, as it was demonstrated that the oral route accounts for the majority of the absorbed aluminum within the human body [169].

However, before discussing NP tissue distributions in more detail, it is important to review the ICP-MS methodology which was used for the quantification of aluminum. As aluminum has a high background presence due to its ubiquitous occurrence, a fairly high aluminum background value was expected. A high background level might result in a non-detection of the treatment derived aluminum. Preliminary experiments showed an organ-dependent variance of the aluminum load. In our opinion, these fluctuations are due to organ matrix effects interfering with the analyte and thus contribute to signal suppression or signal enhancement [65]. The application

of a matrix calibration in combination with a daily response factor led to a compensation of the matrix effects and the daily sensitivity fluctuations of the ICP-MS analysis. With the help of this methodology we were able to quantify the aluminum organ burden in the low µg/g range. The results of our experiments suggest, that a matrix calibration procedure should become the standard methodology for ICP-MS based studies with a high matrix load in order to achieve higher sensitivity and increased robustness in the measurement of ubiquitous elements.

In the context of the study adsorption of both of the aluminum-containing NMs and the ionic $AlCl_3$-$6H_2O$ was demonstrated into secondary organs such as liver, kidney and spleen. Compared to inhalation studies with aluminum NMs, our results show that these NMs maintain an uptake via the oral route [170]. These findings indicate differences between the exposure routes within the same NM group and the relevance of oral uptake for aluminum containing NMs.

A comparison of the aluminum content after exposure to the two aluminum NMs of intestinal tract (colon, duodenum) with the other secondary organs indicates significant differences in the aluminum accumulation behavior. Al_2O_3 showed a concentration dependent increase in the intestinal organs, while for Al^0 NM a dose independent constant distribution ratio could be observed. Furthermore, the distribution within the duodenum and colon also differs significantly between the two aluminum NMs. We attribute these results to an altered agglomeration behavior of the Al_2O_3 NMs, which was further enhanced by physiological conditions such as a pH change between the stomach and intestinal tract. Increased agglomeration thus leads to increased accumulation in the gastrointestinal tract and to a reduction of the aluminum uptake through the gut-blood barrier. These results impressively demonstrate that agglomeration behavior in physiological environments is also a parameter to be accounted for in toxicological studies.

After overcoming the intestinal barrier, both NMs showed a significantly increased blood concentration compared to the ionic $AlCl_3$-$6H_2O$. In this process, the two rather poorly soluble NMs seem to form a protein corona which delays the exchange of aluminum from the blood into the tissue. Such interactions of high molecular weight molecules where already reported by *Sieg et. al.* and further substantiate our results [168].

Between the two NMs we also found differences in organ distribution. In each organ studied, the rod-shaped Al_2O_3 NMs were significantly more abundant than the spherical Al^0 NMs. The shape of the particles seems to have a decisive influence on the retention time in the tissue and the subsequent elimination from the organism. A similar behavior was observed for SiO_2 NMs with a circular or rod-like shape was observed in a 7-day rat study [171]. Possibly, the larger aspect ratio of the rod-shaped NMs may complicate exocytosis, which results in tissue accumulation and retention, an effect we have observed.

Besides the parameters mentioned above, the chemical composition of the NPs may also be one reason for the difference in the translocation behavior. Although both NMs administered are from the group of aluminum-containing NMs, they differ in their structure and aluminum content. While Al_2O_3 is fully oxidized, the Al^0 NMs consist of a core shell structure with a 2-5 nm thick oxide layer. As the surface composition of a NMs is crucial for the expression and structure of the protein corona, these differences in composition might also lead to differences in the formed protein corona. This is supported by *in vitro* studies of CaCo 2 cells that have shown that the protein corona of Al^0 NMs was significantly less complex than the corona of Al_2O_3 NMs [168]. The observed differences in the translocation of the two NMs can also be caused by the differences in the surface composition and the resulting differently complex protein corona.

The findings of this study impressively demonstrate that not only the size of a NM has an influence on interactions with a living organism. The shape, the surface properties and also the agglomeration behavior of a NM can influence the fate of a NM as well. Our data indicate a collective contribution of all physico-chemical characteristics of the chosen NM to the translocation behavior of a NM into secondary organs. The proportion of the respective parameter on the overall translocation rate could not be determined up to now.

Our study shows significant differences in the translocation between aluminum-containing NMs and ionic aluminum. From the available data, it is not possible to draw a conclusion for the limit values. However, it seems reasonable to differentiate between ionic and particulate aluminum in future risk assessment approaches at least for pristine aluminum and aluminum oxide.

The time which is required for such studies has been extensive. In the light of the continuous development of nanotechnology, it is impossible to subject every single NM to a detailed examination. In order to guarantee a safe application of NMs, it is essential to determine the particle properties that have the greatest influence on the uptake and translocation into secondary organs. With grouping strategies and read across approaches it is subsequently possible to close knowledge gaps and ensure the safe application of nanotechnology for humanity and the environment.

However, mechanistic insights about the modes of action of NMs within cellular systems is still lacking in order to successfully implement such grouping approaches. *In vivo* experiments are only of limited suitability for such studies due to the high complexity of the living organism. In the following chapter I would like to discuss the results of the *in vitro* study on the intracellular uptake of aluminum NPs.

4.3. Cellular uptake and distribution of aluminum NMs and the influence of co-exposure to vitamins

Current toxicological research is focused on the effects of single substances, which are tested for toxicological endpoints like LD50 values or no adverse effect levels (NOAEL`s). However, in daily life scenarios the consumer is more likely exposed to mixtures of substances at low-dose levels then to high concentrations of a single compound. Therefore, the consideration of combinatorial effects like additive or synergistic interactions in substance mixtures is a paramount prerequisite for a reliable human health risk assessment. As a model for a multiple exposure setting, *in vitro* systems are usually better suited due to their considerably lower complexity than the classical *in vivo* experiment in order to gain mechanistic insights.

In order to tackle this issue, we investigated potential non-additive combinatorial effects of chemicals in mixtures in the presented study. As already described in chapter 4.2., aluminum and aluminum based NMs are often used in consumer products and especially in cosmetics for example tooth paste or lipsticks [172]. The skin is the major application area for cosmetics; hence we applied the human keratinocyte cell line HaCaT as *in vitro* model for the exposure of Al^0 and Al_2O_3 NMs. In addition to the aluminum-containing NMs from chapter 4.2, retinol (vitamin A) and cholecalciferol (vitamin D3) were co-exposed to HaCaT cells to mimic the exposure of multiple substances to the skin. By understanding the mechanisms of uptake and deposition of NMs in combination with bioactive substances on this process, important insights into safe applications of NMs can be gained.

Within the study ICP-MS was used to quantify the cellular uptake of the different aluminum species. Interestingly, the results showed no influence of the two vitamins investigated on the cellular uptake of Al^0 NM. However, the coexposure of Al_2O_3 NM and each vitamin alone or in combination, resulted in a significantly reduced uptake of the Al_2O_3 NM. As already discussed in chapter 4.2, the differences in the uptake of NMs are most likely the result of the different physico-chemical properties of the materials, which drive the biological interactions. In particular, the complexation and aggregation behavior and the shape of the NM differ

significantly from one another and could therefore be decisive for the preferred endocytosis pathways.

High resolution ToF-SIMS images were acquired to further investigate the absorption behavior of the NMs and the influence of vitamins A and D3 on uptake and metabolic pattern changes thereafter. This technique is particularly suitable for the visualization of chemical distribution patterns with both, a high lateral resolution down to ca. 80nm and a high depth resolution for 3D chemical images down to 10nm. It allows the reconstruction of cells, their metabolites and the absorbed NPs to be visualized qualitatively. 3D reconstruction of single cells demonstrated an altered intracellular distribution pattern for the two applied NMs. While Al^0 NMs are stored in larger agglomerates in distinct areas within the cell, Al_2O_3 uptake resulted in the formation of smaller agglomerates which are localized throughout the cytoplasm. The addition of retinol in the exposure scenarios for Al NMs led in the case of Al^0 to the formation of even larger agglomerates, which were located in close proximity to the cell membrane. Since retinol is known to induce the formation of collagen fibers [173] they could serve as an interaction partner for the Al^0 NMs and be therefore responsible for the observed localization pattern. The addition of vitamin D3 facilitated an enhanced uptake of the Al^0 NMs as well as the formation of smaller agglomerates compared to the treatment with Al^0 alone or in combination with retinol. These findings are in contrast to the results of the exposure of Al_2O_3 in combination with either vitamin which led to a decreased Al uptake into the cell.

A closer analysis of the cell membrane components of the HaCaT cells was carried out to identify the underlying mechanism for the altered uptake and aggregation behavior of NMs within the cell. We identified an increase of diacylglycerols (DAGs) and phosphatidic acids (PAs) following the treatment of either Al NM. These metabolites serve as activators of the protein kinase C signaling pathway [174]. Recent studies have demonstrated an altered shape as well as a strong intra-cellular morphological changes for HaCaT cells after protein kinase C inhibition [175].

The phosphatidylcholine (PC) to phosphatidylethanolamine (PEs) ratio was significantly decreased in cells coexposed with vitamin D3 and Al^0. *In vivo* experiments have shown the suitability of this ratio as biomarker for the induction of a leaky membrane [176]. We therefore concluded that the enhanced uptake of the Al^0 NMs is attributed to a passive diffusion which is

enhanced due to the loss of integrity of the cell membrane. Our data suggest that the interaction of NPs with bioactive substances can strongly influence the uptake and distribution of NPs.

The coexposition of vitamin D3 and Al_2O_3 led to an increased production of dihydroceramides. These sphingolipids are known to increase the rigidity of the plasma membrane. A changed membrane rigidity will subsequently result in a decrease of the passive diffusion through the membrane as well as alterations in the active transport and vesicle formation [177]. These findings are in line with the findings observed in this study, that Al_2O_3 NM uptake was significantly reduced in contrast to Al^0 NM uptake in HaCat cells.

Our results indicate that depending on the chemical composition of the NM and the bioactive substance the cellular response is altered [174]. However, observed changes are not necessarily identical for both NM species as the results of the Vitamin D3 treatment showed. Whether the properties of the NM are responsible for the cellular reaction or perhaps their uptake path could not be clarified in this study. However, the results suggest that the metabolites of the protein kinase-C pathways in combination with membrane lipids are a possible biomarker for NP exposure and uptake.

The results of the study clearly show the need to acquire more data and gain more insights into the co-exposition of nanoparticles and bioactive compounds. The elucidation of the influence of chemical composition and surface chemistry in combination with bioactive compounds on the uptake and the associated change of metabolic cell membrane lipid patterns is of high importance for a reliable risk assessment. Although it was not possible to fully explore the mechanisms involved in the observed metabolic changes, the study shows starting points that can be used for future research. Further mechanistic studies can contribute to the explanation of the principles which drives the observed interactions.

5. Conclusion and Outlook

Nanotechnology has the capability to enhance many aspects of life, from the development of new products to their use in novel disease treatments. However, given the great potential of this technology, there still remains a possible health risk for humans or the environment from this technology.

The comprehensive characterization of NMs is generally regarded as the key for deeper insights of the life-cycle assessment of the material in various environments. A broad range of different characterization techniques have been used to study the physicochemical properties of NMs in detail. However, a universal technique capable of assessing all the characteristics of the material has not yet been developed; in fact, it has become apparent that every technique has its own advantages and shortcomings in terms of NM characterization.

A reference material free reliable quantification sp-ICP-MS method for low concentrated NM was successfully developed within the scope of this work. Through the established setup, a combination of different mathematical approaches for the calculation of material characteristics became feasible. This not only increases the precision of the characterization but also contributes to the validation of the ICP-MS technology.

One of the major challenges in the characterization of NMs is their ability to interact with the environment and thereby undergo alterations. Whether it is the formation of a protein corona, a changed agglomeration behavior or even the dissolution of the material - all these processes present a challenge for current analytical techniques, as they are mostly unable to map these processes in real time. For the future, the focus of method development should be on the establishment and further development of techniques that are able to investigate particles in their respective sample matrix and thus describe their respective state exactly at any time. The development of a size-standard independent particle characterization has laid the foundation to get closer to this goal. Furthermore, the application of a matrix matched calibration approach in

combination with a daily response factor contributes towards a reliable und accurate detection of NMs in complex matrices.

The factors influencing uptake and subsequent translocation into secondary organs of NMs are not yet fully understood. The *in vivo* investigation of aluminum-containing NMs has given us insights into these processes. Our results have shown that despite similar compositions, the biokinetics of NMs can differ greatly. In fact, the agglomeration behavior and shape may also play a decisive role in these processes. Consequently, extrapolation of the particle translocation of NMs is not entirely feasible without clarification of the underlying mechanistic principles. Only when these points have been clarified, risk assessment approaches such as grouping can be successfully applied to all NMs.

Furthermore, low dose study design challenges conventional analytical methods. We therefore implemented a matrix matched calibration process which in combination with a daily response factor led to the sensitive and reliable detection of aluminum in matrix-rich samples. Our results convincingly show that the application of such a procedure for matrix-rich samples enhances the sensitivity of ICP-MS based system and is therefore recommended for such samples.

Cell-based systems can provide important mechanistic insights into the uptake and distribution of NMs. We could show that the exposure of aluminum-containing NMs in combination with bioactive substances leads to changes in lipid composition and the activation of signaling pathways. The combination of Al_2O_3 and vitamin D3 led to a strong reduction of intracellular particle uptake, whereas vitamin D3 and Al^0 exposure resulted in an increased uptake of NMs. While we could demonstrate that the uptake was caused by a shift in the PC to PE ratio and the associated leaky membrane phenomenon the question remains: Which physico-chemical difference of the two aluminum containing NM explains this contrasting behavior? By applying more sophisticated cell culture systems such as organ-on-a-chip platforms, these questions can hopefully be answered for the future and thus contribute to a safer application of NMs.

The work presented here has not only contributed to the advancement in the characterization of NMs, but also provided insights into the interaction of NMs with biological systems, both *in vivo* and *in vitro*. Further studies may adopt and refine these concepts and thus improve the safety of

NM applications. However, there is still a lot of basic research to be done before the toxicity of a NM can be deduced from its physical and chemical properties only. Nevertheless, considering the various opportunities, nanotechnology offers to improve the quality of life. Further efforts should definitely be made to achieve this goal in the future

6. Literature

1. Gupta, N., A.R. Fischer, and L.J. Frewer, *Socio-psychological determinants of public acceptance of technologies: A review.* Public Underst Sci, 2012. **21**(7): p. 782-95.
2. van Wezel, A.P., et al., *Risk analysis and technology assessment in support of technology development: Putting responsible innovation in practice in a case study for nanotechnology.* Integr Environ Assess Manag, 2018. **14**(1): p. 9-16.
3. Lanphier, E., et al., *Don't edit the human germ line.* Nature, 2015. **519**(7544): p. 410-411.
4. Lauer, N.E., J.S. Harkness, and A. Vengosh, *Brine Spills Associated with Unconventional Oil Development in North Dakota.* Environmental Science & Technology, 2016. **50**(10): p. 5389-5397.
5. Keller, K.H., *Nanotechnology and society.* Journal of Nanoparticle Research, 2007. **9**(1): p. 5-10.
6. P Feynman, R., *There's Plenty of Room at the Bottom*. Vol. 1. 1992. 60-66.
7. Custance, O., R. Perez, and S. Morita, *Atomic force microscopy as a tool for atom manipulation.* Nature Nanotechnology, 2009. **4**(12): p. 803-810.
8. Eigler, D.M. and E.K. Schweizer, *Positioning single atoms with a scanning tunnelling microscope.* Nature, 1990. **344**(6266): p. 524-526.
9. Oberdorster, G., E. Oberdorster, and J. Oberdorster, *Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles.* Environ Health Perspect, 2005. **113**(7): p. 823-39.
10. Kumar, N. and S. Kumbhat, *Essentials in Nanoscience and Nanotechnology*. 2016: Wiley.
11. Boholm, M. and R. Arvidsson, *A Definition Framework for the Terms Nanomaterial and Nanoparticle.* NanoEthics, 2016. **10**(1): p. 25-40.
12. Commission Recommendation of 18 October 2011 on the definition of nanomaterial Text with EEA relevance. 2011.
13. Epa, U., *Environmental Protection Agency Nanotechnology White Paper.* EPA, Ed, 2007.
14. Boverhof, D.R., et al., *Comparative assessment of nanomaterial definitions and safety evaluation considerations.* Regulatory Toxicology and Pharmacology, 2015. **73**(1): p. 137-150.
15. Horikoshi, S. and N. Serpone, *Microwaves in nanoparticle synthesis: fundamentals and applications*. 2013: John Wiley & Sons.
16. Saner, M. and A. Stoklosa, *Commercial, Societal and Administrative Benefits from the Analysis and Clarification of Definitions: The Case of Nanomaterials.* Creativity and Innovation Management, 2013. **22**(1): p. 26-36.
17. Maynard, A.D., *Don't define nanomaterials.* Nature, 2011. **475**(7354): p. 31-31.
18. Handy, R.D., et al., *The ecotoxicology and chemistry of manufactured nanoparticles.* Ecotoxicology, 2008. **17**(4): p. 287-314.
19. Alkilany, A.M. and C.J. Murphy, *Toxicity and cellular uptake of gold nanoparticles: what we have learned so far?* Journal of nanoparticle research : an interdisciplinary forum for nanoscale science and technology, 2010. **12**(7): p. 2313-2333.
20. Turner, M., et al., *Selective oxidation with dioxygen by gold nanoparticle catalysts derived from 55-atom clusters.* Nature, 2008. **454**(7207): p. 981-3.
21. Khan, I., K. Saeed, and I. Khan, *Nanoparticles: Properties, applications and toxicities.* Arabian Journal of Chemistry, 2019. **12**(7): p. 908-931.
22. Garcia, M.A., *Surface plasmons in metallic nanoparticles: fundamentals and applications.* Journal of Physics D: Applied Physics, 2011. **44**(28): p. 283001.
23. Kreibig, U. and M. Vollmer, *Optical properties of metal clusters*. Vol. 25. 2013: Springer Science & Business Media.
24. Barnes, W.L., A. Dereux, and T.W. Ebbesen, *Surface plasmon subwavelength optics.* Nature, 2003. **424**(6950): p. 824-830.

25. El-Sayed, I.H., X. Huang, and M.A. El-Sayed, *Surface plasmon resonance scattering and absorption of anti-EGFR antibody conjugated gold nanoparticles in cancer diagnostics: applications in oral cancer.* Nano letters, 2005. **5**(5): p. 829-834.
26. Loo, C., et al., *Immunotargeted nanoshells for integrated cancer imaging and therapy.* Nano letters, 2005. **5**(4): p. 709-711.
27. Jain, P.K., et al., *Calculated absorption and scattering properties of gold nanoparticles of different size, shape, and composition: applications in biological imaging and biomedicine.* The journal of physical chemistry B, 2006. **110**(14): p. 7238-7248.
28. Jain, P.K., et al., *Review of Some Interesting Surface Plasmon Resonance-enhanced Properties of Noble Metal Nanoparticles and Their Applications to Biosystems.* Plasmonics, 2007. **2**(3): p. 107-118.
29. Haes, A.J., et al., *A Localized Surface Plasmon Resonance Biosensor: First Steps toward an Assay for Alzheimer's Disease.* Nano Letters, 2004. **4**(6): p. 1029-1034.
30. Tan, Y.N., et al., *Sensing of Transcription Factor through Controlled-Assembly of Metal Nanoparticles Modified with Segmented DNA Elements.* ACS Nano, 2010. **4**(9): p. 5101-5110.
31. Stewart, M.E., et al., *Nanostructured plasmonic sensors.* Chemical reviews, 2008. **108**(2): p. 494-521.
32. Chung, T., et al., *Plasmonic nanostructures for nano-scale bio-sensing.* Sensors (Basel), 2011. **11**(11): p. 10907-29.
33. Na, H.B., I.C. Song, and T. Hyeon, *Inorganic Nanoparticles for MRI Contrast Agents.* Advanced Materials, 2009. **21**(21): p. 2133-2148.
34. Chomoucka, J., et al., *Magnetic nanoparticles and targeted drug delivering.* Pharmacological Research, 2010. **62**(2): p. 144-149.
35. Reiss, G. and A. Hütten, *Applications beyond data storage.* Nature Materials, 2005. **4**(10): p. 725-726.
36. Laurent, S., et al., *Magnetic iron oxide nanoparticles: synthesis, stabilization, vectorization, physicochemical characterizations, and biological applications.* Chemical reviews, 2008. **108**(6): p. 2064-2110.
37. Gungor, A.A., H. Nadaroglu, and N. Celebi, *Fenton process for basic red 9 degradation: immobilized apolaccase on a nanomagnetite system.* Environmental and Experimental Biology, 2014. **12**: p. 121-129.
38. Hua, M., et al., *Heavy metal removal from water/wastewater by nanosized metal oxides: a review.* Journal of hazardous materials, 2012. **211**: p. 317-331.
39. Fan, J. and Y. Gao, *Nanoparticle-supported catalysts and catalytic reactions – a mini-review.* Journal of Experimental Nanoscience, 2006. **1**(4): p. 457-475.
40. McCarty, G. and P. Weiss, *Scanning probe studies of single nanostructures.* Chemical reviews, 1999. **99**(7): p. 1983-1990.
41. Creus, J., et al., *Ruthenium Nanoparticles for Catalytic Water Splitting.* ChemSusChem, 2019. **12**(12): p. 2493-2514.
42. Guo, D., G. Xie, and J. Luo, *Mechanical properties of nanoparticles: basics and applications.* Journal of Physics D: Applied Physics, 2013. **47**(1): p. 013001.
43. Taofang, Z. and S. Thomas, *Size effect of nanoparticles in chemical mechanical polishing - a transient model.* IEEE Transactions on Semiconductor Manufacturing, 2005. **18**(4): p. 655-663.
44. Gebauer, J.S., et al., *Impact of the Nanoparticle–Protein Corona on Colloidal Stability and Protein Structure.* Langmuir, 2012. **28**(25): p. 9673-9679.
45. Leszczynski, J., *Nano meets bio at the interface.* Nature Nanotechnology, 2010. **5**(9): p. 633-634.
46. Nguyen, V.H. and B.J. Lee, *Protein corona: a new approach for nanomedicine design.* Int J Nanomedicine, 2017. **12**: p. 3137-3151.
47. Barbero, F., et al., *Formation of the Protein Corona: The Interface between Nanoparticles and the Immune System.* Seminars in Immunology, 2017. **34**: p. 52-60.

48. Monopoli, M.P., et al., *Physical−Chemical Aspects of Protein Corona: Relevance to in Vitro and in Vivo Biological Impacts of Nanoparticles.* Journal of the American Chemical Society, 2011. **133**(8): p. 2525-2534.
49. Walkey, C.D., et al., *Nanoparticle Size and Surface Chemistry Determine Serum Protein Adsorption and Macrophage Uptake.* Journal of the American Chemical Society, 2012. **134**(4): p. 2139-2147.
50. Peer, D., et al., *Nanocarriers as an emerging platform for cancer therapy.* Nature Nanotechnology, 2007. **2**(12): p. 751-760.
51. Powers, K.W., et al., *Research Strategies for Safety Evaluation of Nanomaterials. Part VI. Characterization of Nanoscale Particles for Toxicological Evaluation.* Toxicological Sciences, 2006. **90**(2): p. 296-303.
52. Oberdörster, G., et al., *Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy.* Particle and fibre toxicology, 2005. **2**(1): p. 8.
53. Powers, K.W., et al., *Characterization of the size, shape, and state of dispersion of nanoparticles for toxicological studies.* Nanotoxicology, 2007. **1**(1): p. 42-51.
54. Laux, P., et al., *Nanomaterials: certain aspects of application, risk assessment and risk communication.* Archives of toxicology, 2018. **92**(1): p. 121-141.
55. Fissan, H., et al., *Comparison of different characterization methods for nanoparticle dispersions before and after aerosolization.* Analytical Methods, 2014. **6**(18): p. 7324-7334.
56. Lim, J., et al., *Characterization of magnetic nanoparticle by dynamic light scattering.* Nanoscale research letters, 2013. **8**(1): p. 381-381.
57. Liu, X., et al., *A One-Step Homogeneous Immunoassay for Cancer Biomarker Detection Using Gold Nanoparticle Probes Coupled with Dynamic Light Scattering.* Journal of the American Chemical Society, 2008. **130**(9): p. 2780-2782.
58. Domingos, R.F., et al., *Characterizing manufactured nanoparticles in the environment: multimethod determination of particle sizes.* Environmental science & technology, 2009. **43**(19): p. 7277-7284.
59. Krause, B., et al., *Characterization of aluminum, aluminum oxide and titanium dioxide nanomaterials using a combination of methods for particle surface and size analysis.* RSC Advances, 2018. **8**(26): p. 14377-14388.
60. Hassellöv, M., et al., *Nanoparticle analysis and characterization methodologies in environmental risk assessment of engineered nanoparticles.* Ecotoxicology, 2008. **17**(5): p. 344-361.
61. Filipe, V., A. Hawe, and W. Jiskoot, *Critical Evaluation of Nanoparticle Tracking Analysis (NTA) by NanoSight for the Measurement of Nanoparticles and Protein Aggregates.* Pharmaceutical Research, 2010. **27**(5): p. 796-810.
62. Gottschalk, F., et al., *Modeled Environmental Concentrations of Engineered Nanomaterials (TiO2, ZnO, Ag, CNT, Fullerenes) for Different Regions.* Environmental Science & Technology, 2009. **43**(24): p. 9216-9222.
63. Pace, H.E., et al., *Single Particle Inductively Coupled Plasma-Mass Spectrometry: A Performance Evaluation and Method Comparison in the Determination of Nanoparticle Size.* Environmental Science & Technology, 2012. **46**(22): p. 12272-12280.
64. Mozhayeva, D. and C. Engelhard, *A critical review of single particle inductively coupled plasma mass spectrometry – A step towards an ideal method for nanomaterial characterization.* Journal of Analytical Atomic Spectrometry, 2020.
65. Rosenkranz, D., et al., *Improved validation for single particle ICP-MS analysis using a pneumatic nebulizer / microdroplet generator sample introduction system for multi-mode nanoparticle determination.* Analytica Chimica Acta, 2020. **1099**: p. 16-25.
66. Mitrano, D.M., et al., *Detecting nanoparticulate silver using single-particle inductively coupled plasma–mass spectrometry.* Environmental Toxicology and Chemistry, 2012. **31**(1): p. 115-121.
67. Degueldre, C., P.Y. Favarger, and C. Bitea, *Zirconia colloid analysis by single particle inductively coupled plasma–mass spectrometry.* Analytica Chimica Acta, 2004. **518**(1): p. 137-142.

68. Mattarozzi, M., et al., *Analytical approaches for the characterization and quantification of nanoparticles in food and beverages.* Analytical and Bioanalytical Chemistry, 2017. **409**(1): p. 63-80.
69. Degueldre, C. and P.Y. Favarger, *Colloid analysis by single particle inductively coupled plasma-mass spectroscopy: a feasibility study.* Colloids and Surfaces A: Physicochemical and Engineering Aspects, 2003. **217**(1): p. 137-142.
70. Balnois, E., et al., *Environmental Colloids and Particles: Behaviour, Structure, and Characterization*. 2007, Wiley.
71. Su, D., *Advanced electron microscopy characterization of nanomaterials for catalysis.* Green Energy & Environment, 2017. **2**(2): p. 70-83.
72. Buzea, C., I.I. Pacheco, and K. Robbie, *Nanomaterials and nanoparticles: Sources and toxicity.* Biointerphases, 2007. **2**(4): p. MR17-MR71.
73. Sohaebuddin, S.K., et al., *Nanomaterial cytotoxicity is composition, size, and cell type dependent.* Particle and Fibre Toxicology, 2010. **7**(1): p. 22.
74. Winnik, F.M. and D. Maysinger, *Quantum Dot Cytotoxicity and Ways To Reduce It.* Accounts of Chemical Research, 2013. **46**(3): p. 672-680.
75. Harper, S., et al., *In vivo biodistribution and toxicity depends on nanomaterial composition, size, surface functionalisation and route of exposure.* Journal of Experimental Nanoscience, 2008. **3**(3): p. 195-206.
76. Fabricius, A.-L., et al., *ICP-MS-based characterization of inorganic nanoparticles--sample preparation and off-line fractionation strategies.* Analytical and bioanalytical chemistry, 2014. **406**(2): p. 467-479.
77. Hunt, J.A. and D.B. Williams, *Electron energy-loss spectrum-imaging.* Ultramicroscopy, 1991. **38**(1): p. 47-73.
78. Li, W. and C. Ni, *Electron Energy Loss Spectroscopy (EELS)*, in *Encyclopedia of Tribology*, Q.J. Wang and Y.-W. Chung, Editors. 2013, Springer US: Boston, MA. p. 940-945.
79. Hawkes, P.W. and J.C. Spence, *Science of microscopy*. 2008: Springer Science & Business Media.
80. Lanza, M., *Conductive Atomic Force Microscopy: Applications in Nanomaterials*. 2017: John Wiley & Sons.
81. Modena, M.M., et al., *Nanoparticle Characterization: What to Measure?* Advanced Materials, 2019. **31**(32): p. 1901556.
82. Cervellino, A., et al., *X-ray and Neutron Techniques for Nanomaterials Characterization.* Springer-Verlag GmbH Germany: Berlin, Germany, 2016: p. 545-608.
83. Brison, J., et al., *ToF-SIMS imaging and depth profiling of HeLa cells treated with bromodeoxyuridine.* Surface and interface analysis : SIA, 2011. **43**(1-2): p. 354-357.
84. Singh, A.V., et al., *ToF-SIMS 3D imaging unveils important insights on the cellular microenvironment during biomineralization of gold nanostructures.* Scientific Reports, 2020. **10**(1): p. 261.
85. Lamon, L., et al., *Grouping of nanomaterials to read-across hazard endpoints: a review.* Nanotoxicology, 2019. **13**(1): p. 100-118.
86. Giusti, A., et al., *Nanomaterial grouping: Existing approaches and future recommendations.* NanoImpact, 2019. **16**: p. 100182.
87. Lynch, I., C. Weiss, and E. Valsami-Jones, *A strategy for grouping of nanomaterials based on key physico-chemical descriptors as a basis for safer-by-design NMs.* Nano Today, 2014. **9**(3): p. 266-270.
88. Giese, B., et al., *Risks, Release and Concentrations of Engineered Nanomaterial in the Environment.* Scientific Reports, 2018. **8**(1): p. 1565.
89. Frohlich, E. and E. Roblegg, *Oral uptake of nanoparticles: human relevance and the role of in vitro systems.* Arch Toxicol, 2016. **90**(10): p. 2297-314.
90. Alvarez-Roman, R., et al., *Skin penetration and distribution of polymeric nanoparticles.* J Control Release, 2004. **99**(1): p. 53-62.

91. Schneider, M., et al., *Nanoparticles and their interactions with the dermal barrier.* Dermato-Endocrinology, 2009. **1**(4): p. 197-206.
92. Langbein, L., et al., *Tight junctions and compositionally related junctional structures in mammalian stratified epithelia and cell cultures derived therefrom.* Eur J Cell Biol, 2002. **81**(8): p. 419-35.
93. Nohynek, G.J., et al., *Grey Goo on the Skin? Nanotechnology, Cosmetic and Sunscreen Safety.* Critical Reviews in Toxicology, 2007. **37**(3): p. 251-277.
94. Yu, B.D., A. Mukhopadhyay, and C. Wong, *Skin and hair: models for exploring organ regeneration.* Hum Mol Genet, 2008. **17**(R1): p. R54-9.
95. Jatana, S. and L.A. DeLouise, *Understanding engineered nanomaterial skin interactions and the modulatory effects of ultraviolet radiation skin exposure.* Wiley interdisciplinary reviews. Nanomedicine and nanobiotechnology, 2014. **6**(1): p. 61-79.
96. Kertész, Z., et al., *Nuclear microprobe study of TiO2-penetration in the epidermis of human skin xenografts.* Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms, 2005. **231**(1): p. 280-285.
97. Crosera, M., et al., *Nanoparticle dermal absorption and toxicity: a review of the literature.* International Archives of Occupational and Environmental Health, 2009. **82**(9): p. 1043-1055.
98. Trop, M., et al., *Silver-coated dressing acticoat caused raised liver enzymes and argyria-like symptoms in burn patient.* J Trauma, 2006. **60**(3): p. 648-52.
99. Gulson, B., et al., *Small Amounts of Zinc from Zinc Oxide Particles in Sunscreens Applied Outdoors Are Absorbed through Human Skin.* Toxicological Sciences, 2010. **118**(1): p. 140-149.
100. Tentschert, J., et al., *Organ burden of inhaled nanoceria in a 2-year low-dose exposure study: dump or depot?* Nanotoxicology, 2020. **14**(4): p. 554-576.
101. Carvalho, T.C., J.I. Peters, and R.O. Williams, *Influence of particle size on regional lung deposition – What evidence is there?* International Journal of Pharmaceutics, 2011. **406**(1): p. 1-10.
102. Patton, J.S., et al., *The particle has landed--characterizing the fate of inhaled pharmaceuticals.* J Aerosol Med Pulm Drug Deliv, 2010. **23 Suppl 2**: p. S71-87.
103. Geiser, M. and W.G. Kreyling, *Deposition and biokinetics of inhaled nanoparticles.* Particle and Fibre Toxicology, 2010. **7**(1): p. 2.
104. Yang, W., J.I. Peters, and R.O. Williams, 3rd, *Inhaled nanoparticles--a current review.* Int J Pharm, 2008. **356**(1-2): p. 239-47.
105. Paranjpe, M. and C.C. Müller-Goymann, *Nanoparticle-mediated pulmonary drug delivery: a review.* International journal of molecular sciences, 2014. **15**(4): p. 5852-5873.
106. Hu, G. and J.W. Christman, *Editorial: Alveolar Macrophages in Lung Inflammation and Resolution.* Frontiers in Immunology, 2019. **10**(2275).
107. Schleh, C. and J.M. Hohlfeld, *Interaction of nanoparticles with the pulmonary surfactant system.* Inhalation Toxicology, 2009. **21**(sup1): p. 97-103.
108. Semmler, M., et al., *Long-term clearance kinetics of inhaled ultrafine insoluble iridium particles from the rat lung, including transient translocation into secondary organs.* Inhal Toxicol, 2004. **16**(6-7): p. 453-9.
109. Geiser, M., et al., *The role of macrophages in the clearance of inhaled ultrafine titanium dioxide particles.* Am J Respir Cell Mol Biol, 2008. **38**(3): p. 371-6.
110. Miller, M.R., et al., *Inhaled Nanoparticles Accumulate at Sites of Vascular Disease.* ACS Nano, 2017. **11**(5): p. 4542-4552.
111. Raftis, J.B. and M.R. Miller, *Nanoparticle translocation and multi-organ toxicity: A particularly small problem.* Nano Today, 2019. **26**: p. 8-12.
112. Kreyling, W.G., et al., *Air–Blood Barrier Translocation of Tracheally Instilled Gold Nanoparticles Inversely Depends on Particle Size.* ACS Nano, 2014. **8**(1): p. 222-233.
113. Kreyling, W.G., et al., *In vitro and in vivo interactions of selected nanoparticles with rodent serum proteins and their consequences in biokinetics.* Beilstein Journal of Nanotechnology, 2014. **5**: p. 1699-1711.

114. McClements, D.J. and H. Xiao, *Is nano safe in foods? Establishing the factors impacting the gastrointestinal fate and toxicity of organic and inorganic food-grade nanoparticles.* npj Science of Food, 2017. **1**(1): p. 6.
115. Bott, J., A. Störmer, and R. Franz, *A model study into the migration potential of nanoparticles from plastics nanocomposites for food contact.* Food Packaging and Shelf Life, 2014. **2**(2): p. 73-80.
116. Maia, J., et al., *Determination of key diffusion and partition parameters and their use in migration modelling of benzophenone from low-density polyethylene (LDPE) into different foodstuffs.* Food Addit Contam Part A Chem Anal Control Expo Risk Assess, 2016. **33**(4): p. 715-24.
117. Sieg, H., et al., *Impact of an Artificial Digestion Procedure on Aluminum-Containing Nanomaterials.* Langmuir, 2017. **33**(40): p. 10726-10735.
118. Abdelkhaliq, A., et al., *Impact of in vitro digestion on gastrointestinal fate and uptake of silver nanoparticles with different surface modifications.* Nanotoxicology, 2020. **14**(1): p. 111-126.
119. McCracken, C., et al., *Oxidative stress-mediated inhibition of intestinal epithelial cell proliferation by silver nanoparticles.* Toxicol In Vitro, 2015. **29**(7): p. 1793-808.
120. Lai, S.K., Y.Y. Wang, and J. Hanes, *Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues.* Adv Drug Deliv Rev, 2009. **61**(2): p. 158-71.
121. des Rieux, A., et al., *Nanoparticles as potential oral delivery systems of proteins and vaccines: a mechanistic approach.* J Control Release, 2006. **116**(1): p. 1-27.
122. Florence, A.T., *The Oral Absorption of Micro- and Nanoparticulates: Neither Exceptional Nor Unusual.* Pharmaceutical Research, 1997. **14**(3): p. 259-266.
123. Florence, A.T., *Nanoparticle uptake by the oral route: Fulfilling its potential?* Drug Discovery Today: Technologies, 2005. **2**(1): p. 75-81.
124. Li, M., et al., *Physiologically based pharmacokinetic modeling of nanoparticles.* ACS nano, 2010. **4**(11): p. 6303-6317.
125. Hante, N.K., C. Medina, and M.J. Santos-Martinez, *Effect on Platelet Function of Metal-Based Nanoparticles Developed for Medical Applications.* Frontiers in Cardiovascular Medicine, 2019. **6**(139).
126. Longmire, M., P.L. Choyke, and H. Kobayashi, *Clearance properties of nano-sized particles and molecules as imaging agents: considerations and caveats.* Nanomedicine (London, England), 2008. **3**(5): p. 703-717.
127. Hamidi, M., A. Azadi, and P. Rafiei, *Pharmacokinetic consequences of pegylation.* Drug Deliv, 2006. **13**(6): p. 399-409.
128. Arvizo, R.R., et al., *Modulating Pharmacokinetics, Tumor Uptake and Biodistribution by Engineered Nanoparticles.* PLOS ONE, 2011. **6**(9): p. e24374.
129. Lin, Z., N.A. Monteiro-Riviere, and J.E. Riviere, *Pharmacokinetics of metallic nanoparticles.* Wiley Interdiscip Rev Nanomed Nanobiotechnol, 2015. **7**(2): p. 189-217.
130. Behzadi, S., et al., *Cellular uptake of nanoparticles: journey inside the cell.* Chemical Society reviews, 2017. **46**(14): p. 4218-4244.
131. Kumari, S., S. Mg, and S. Mayor, *Endocytosis unplugged: multiple ways to enter the cell.* Cell Res, 2010. **20**(3): p. 256-75.
132. Aderem, A. and D.M. Underhill, *Mechanisms of phagocytosis in macrophages.* Annu Rev Immunol, 1999. **17**: p. 593-623.
133. Hillaireau, H. and P. Couvreur, *Nanocarriers' entry into the cell: relevance to drug delivery.* Cell Mol Life Sci, 2009. **66**(17): p. 2873-96.
134. Tabata, Y. and Y. Ikada, *Effect of the size and surface charge of polymer microspheres on their phagocytosis by macrophage.* Biomaterials, 1988. **9**(4): p. 356-62.
135. Couvreur, P. and C. Vauthier, *Nanotechnology: intelligent design to treat complex disease.* Pharm Res, 2006. **23**(7): p. 1417-50.
136. Conner, S.D. and S.L. Schmid, *Regulated portals of entry into the cell.* Nature, 2003. **422**(6927): p. 37-44.

137. Doherty, G.J. and H.T. McMahon, *Mechanisms of Endocytosis.* Annual Review of Biochemistry, 2009. **78**(1): p. 857-902.
138. Harush-Frenkel, O., et al., *Targeting of nanoparticles to the clathrin-mediated endocytic pathway.* Biochem Biophys Res Commun, 2007. **353**(1): p. 26-32.
139. Panyam, J., et al., *Rapid endo-lysosomal escape of poly(DL-lactide-co-glycolide) nanoparticles: implications for drug and gene delivery.* Faseb j, 2002. **16**(10): p. 1217-26.
140. Zhao, J. and M.H. Stenzel, *Entry of nanoparticles into cells: the importance of nanoparticle properties.* Polymer Chemistry, 2018. **9**(3): p. 259-272.
141. Rejman, J., M. Conese, and D. Hoekstra, *Gene Transfer by Means of Lipo- and Polyplexes: Role of Clathrin and Caveolae-Mediated Endocytosis.* Journal of Liposome Research, 2006. **16**(3): p. 237-247.
142. Sakhtianchi, R., et al., *Exocytosis of nanoparticles from cells: Role in cellular retention and toxicity.* Advances in Colloid and Interface Science, 2013. **201-202**: p. 18-29.
143. Stayton, I., et al., *Study of uptake and loss of silica nanoparticles in living human lung epithelial cells at single cell level.* Analytical and Bioanalytical Chemistry, 2009. **394**(6): p. 1595-1608.
144. Salaün, C., D.J. James, and L.H. Chamberlain, *Lipid rafts and the regulation of exocytosis.* Traffic, 2004. **5**(4): p. 255-64.
145. Krol, S., et al., *Therapeutic benefits from nanoparticles: the potential significance of nanoscience in diseases with compromise to the blood brain barrier.* Chem Rev, 2013. **113**(3): p. 1877-903.
146. Soo Choi, H., et al., *Renal clearance of quantum dots.* Nature Biotechnology, 2007. **25**(10): p. 1165-1170.
147. Haute, D.V. and J.M. Berlin, *Challenges in realizing selectivity for nanoparticle biodistribution and clearance: lessons from gold nanoparticles.* Therapeutic delivery, 2017. **8**(9): p. 763-774.
148. Zhang, Y.-N., et al., *Nanoparticle–liver interactions: Cellular uptake and hepatobiliary elimination.* Journal of Controlled Release, 2016. **240**: p. 332-348.
149. Huang, Y.-W., M. Cambre, and H.-J. Lee, *The Toxicity of Nanoparticles Depends on Multiple Molecular and Physicochemical Mechanisms.* International journal of molecular sciences, 2017. **18**(12): p. 2702.
150. Oberdörster, G., et al., *Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy.* Part Fibre Toxicol, 2005. **2**: p. 8.
151. Schmid, O. and T. Stoeger, *Surface area is the biologically most effective dose metric for acute nanoparticle toxicity in the lung.* Journal of Aerosol Science, 2016. **99**: p. 133-143.
152. Shin, S.W., I.H. Song, and S.H. Um, *Role of Physicochemical Properties in Nanoparticle Toxicity.* Nanomaterials (Basel, Switzerland), 2015. **5**(3): p. 1351-1365.
153. Luanpitpong, S., L. Wang, and Y. Rojanasakul, *The effects of carbon nanotubes on lung and dermal cellular behaviors.* Nanomedicine (London, England), 2014. **9**(6): p. 895-912.
154. Nel, A., et al., *Toxic potential of materials at the nanolevel.* Science, 2006. **311**(5761): p. 622-7.
155. Xia, T., et al., *Comparison of the abilities of ambient and manufactured nanoparticles to induce cellular toxicity according to an oxidative stress paradigm.* Nano Lett, 2006. **6**(8): p. 1794-807.
156. Han, D., E. Williams, and E. Cadenas, *Mitochondrial respiratory chain-dependent generation of superoxide anion and its release into the intermembrane space.* Biochem J, 2001. **353**(Pt 2): p. 411-6.
157. Lin, W., et al., *Toxicity of cerium oxide nanoparticles in human lung cancer cells.* Int J Toxicol, 2006. **25**(6): p. 451-7.
158. Lin, W., et al., *Toxicity of nano- and micro-sized ZnO particles in human lung epithelial cells.* Journal of Nanoparticle Research, 2009. **11**(1): p. 25-39.
159. Saptarshi, S.R., A. Duschl, and A.L. Lopata, *Interaction of nanoparticles with proteins: relation to bio-reactivity of the nanoparticle.* Journal of nanobiotechnology, 2013. **11**: p. 26-26.
160. Wagner, S.C., et al., *Nanoparticle-induced folding and fibril formation of coiled-coil-based model peptides.* Small, 2010. **6**(12): p. 1321-8.
161. Deng, Z.J., et al., *Nanoparticle-induced unfolding of fibrinogen promotes Mac-1 receptor activation and inflammation.* Nat Nanotechnol, 2011. **6**(1): p. 39-44.

162. Martinolich, A.J., et al., *Structural and Functional Effects of Cu Metalloprotein-Driven Silver Nanoparticle Dissolution.* Environmental Science & Technology, 2012. **46**(11): p. 6355-6362.
163. Sun, H., et al., *Gold Nanoparticle-Induced Cell Death and Potential Applications in Nanomedicine.* International journal of molecular sciences, 2018. **19**(3): p. 754.
164. Chatterjee, N. and G.C. Walker, *Mechanisms of DNA damage, repair, and mutagenesis.* Environmental and molecular mutagenesis, 2017. **58**(5): p. 235-263.
165. Sharifi, S., et al., *Toxicity of nanomaterials.* Chemical Society Reviews, 2012. **41**(6): p. 2323-2343.
166. Ramkorun-Schmidt, B., et al., *Investigation of a Combined Microdroplet Generator and Pneumatic Nebulization System for Quantitative Determination of Metal-Containing Nanoparticles Using ICPMS.* Analytical Chemistry, 2015. **87**(17): p. 8687-8694.
167. Gschwind, S., M.d.L. Aja Montes, and D. Günther, *Comparison of sp-ICP-MS and MDG-ICP-MS for the determination of particle number concentration.* Analytical and Bioanalytical Chemistry, 2015. **407**(14): p. 4035-4044.
168. Sieg, H., et al., *Uptake and molecular impact of aluminum-containing nanomaterials on human intestinal caco-2 cells.* Nanotoxicology, 2018. **12**(9): p. 992-1013.
169. Krewski, D., et al., *Human health risk assessment for aluminium, aluminium oxide, and aluminium hydroxide.* Journal of Toxicology and Environmental Health, Part B, 2007. **10**(S1): p. 1-269.
170. Pauluhn, J., *Pulmonary toxicity and fate of agglomerated 10 and 40 nm aluminum oxyhydroxides following 4-week inhalation exposure of rats: toxic effects are determined by agglomerated, not primary particle size.* Toxicological Sciences, 2009. **109**(1): p. 152-167.
171. Zhao, Y., et al., *A comparison between sphere and rod nanoparticles regarding their in vivo biological behavior and pharmacokinetics.* Scientific Reports, 2017. **7**(1): p. 4131.
172. Fytianos, G., A. Rahdar, and G.Z. Kyzas, *Nanomaterials in Cosmetics: Recent Updates.* Nanomaterials (Basel, Switzerland), 2020. **10**(5): p. 979.
173. Kandamachira, A., et al., *Collagen-nanoparticle interactions: type I collagen stabilization using functionalized nanoparticles.* Soft Materials, 2015. **13**(1): p. 59-65.
174. Kriegel, F.L., et al., *The Vitamin A and D Exposure of Cells Affects the Intracellular Uptake of Aluminum Nanomaterials and its Agglomeration Behavior: A Chemo-Analytic Investigation.* Int J Mol Sci, 2020. **21**(4).
175. Hegemann, L., et al., *Changes of epidermal cell morphology and keratin expression induced by inhibitors of protein kinase C.* Journal of dermatological science, 1992. **3**(2): p. 103-110.
176. Li, Z., et al., *The ratio of phosphatidylcholine to phosphatidylethanolamine influences membrane integrity and steatohepatitis.* Cell metabolism, 2006. **3**(5): p. 321-331.
177. Stiban, J., D. Fistere, and M. Colombini, *Dihydroceramide hinders ceramide channel formation: implications on apoptosis.* Apoptosis, 2006. **11**(5): p. 773-780.

7. List of publications

7.1. Publications integrated in this thesis

Rosenkranz, D.; Kriegel, F.L.; Mavrakis, E.; Pergantis, S.A.; Reichardt, P.; Tentschert, J.; Jakubowski, N.; Laux, P.; Panne, U.; Luch, A. Improved validation for single particle ICP-MS analysis using a pneumatic nebulizer / microdroplet generator sample introduction system for multi-mode NP determination. Analytica Chimica Acta 2020, 1099, 16-25, doi:https://doi.org/10.1016/j.aca.2019.11.043.

Krause, B.C.; Kriegel, F.L.; Rosenkranz, D.; Dreiack, N.; Tentschert, J.; Jungnickel, H.; Jalili, P.; Fessard, V.; Laux, P.; Luch, A. Aluminum and aluminum oxide NMs uptake after oral exposure - a comparative study. *Sci Rep-Uk* **2020**, *10*, 2698, doi:10.1038/s41598-020-59710-z.

Kriegel, F.L.; Reichardt, P.; Krause, B.C.; Singh, A.V.; Tentschert, J.; Laux, P.; Jungnickel, H.; Luch, A. The Vitamin A and D Exposure of Cells Affects the Intracellular Uptake of Aluminum NMs and its Agglomeration Behavior: A Chemo-Analytic Investigation. Int J Mol Sci 2020, 21, doi:10.3390/ijms21041278.

7.2. Other publications

Kriegel, F. L.; Köhler, R.; Bayat-Sarmadi, J.; Bayerl, S.; Hauser, A. E.; Niesner, R.; Luch, A.; Cseresnyes, Z. Cell shape characterization and classification with discrete Fourier transforms and self-organizing maps. Cytometry Part A 2017, 93, 323-333, doi:10.1002/cyto.a.23279.

Kriegel, F. L., Köhler, R., Bayat-Sarmadi, J., Bayerl, S., Hauser, A. E., Niesner, R., Luch, A., Cseresnyes, Z. Morphology-Based Distinction Between Healthy and Pathological Cells Utilizing Fourier Transforms and Self-Organizing Maps. *J. Vis. Exp.* (140), e58543, doi:10.3791/58543 (2018).

Hachenberger, Y.U.; Rosenkranz, D.; Kriegel, F.L.; Krause, B.; Matschaß, R.; Reichardt, P.; Tentschert, J.; Laux, P.; Jakubowski, N.; Panne, U., et al. Tackling Complex Analytical Tasks: An ISO/TS-Based Validation Approach for Hydrodynamic Chromatography Single Particle Inductively Coupled Plasma Mass Spectrometry. *Materials (Basel)* **2020**, *13*, 1447, doi:10.3390/ma13061447.

Singh, A. V., Ansari, M. H. D., Rosenkranz, D., Maharjan, R. S., Kriegel, F. L., Gandhi, K., Kanase, A., Singh, R., Laux, P., Luch, A., Artificial Intelligence and Machine Learning in Computational Nanotoxicology: Unlocking and Empowering Nanomedicine. Adv. Healthcare Mater. 2020, 1901862. https://doi.org/10.1002/adhm.201901862

Rosenkranz, D., Kriegel, F. L., Mavrakis, E., Pergantis, S. A., Reichardt, P., Tentschert, J., Jakubowski, N., Laux, P., Panne, U., Luch, A. Versatile Dual-Inlet Sample Introduction System for Multi-Mode Single Particle Inductively Coupled Plasma Mass Spectrometry Analysis and Validation. J. Vis. Exp. (163), e61653, doi:10.3791/61653 (2020).

Annex I

Supporting Information

Improved Validation for Single Particle ICP-MS Analysis Using a Pneumatic Nebulizer / Microdroplet Generator Sample Introduction System for Multi-mode NP Determination

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Annex II

Supporting Information

Aluminum and aluminum oxide NMs uptake after oral exposure - a comparative study

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Annex III

Supporting Information

The Vitamin A and D Exposure of Cells Affects the Intracellular Uptake of Aluminum NMs and Its Agglomeration Behavior: A Chemo-Analytic Investigation

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