Aus dem Institut für Tierpathologie
des Fachbereichs Veterinärmedizin der Freien Universität Berlin

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Silica Nanoparticles in Mouse Models of Skin Diseases:

Local Penetration, Systemic Distribution and

Effect on Allergic Contact Dermatitis

Inaugural-Dissertation
zur Erlangung des Grades eines

Doctor of Philosophy (Ph.D.)

in Biomedical Sciences

an der

Freien Universität Berlin

vorgelegt von

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Tierärztin aus Burg bei Magdeburg

Berlin 2014

Journal-Nr.: 3760

Gedruckt mit Genehmigung des Fachbereichs Veterinärmedizin

der Freien Universität Berlin

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Deskriptoren (nach CAB-Thesaurus):
mice, animal models, nanoparticles, hypersensitivity, dermatitis, fluorescence microscopy, transmission electron microscopy, immunohistochemistry, immunofluorescence, ELISA

Tag der Promotion: 05.12.2014

Bibliografische Information der *Deutschen Nationalbibliothek*
Die Deutsche Nationalbibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliografie; detaillierte bibliografische Daten sind im Internet über <http://dnb.ddb.de> abrufbar.

ISBN: 978-3-86387-545-9
Zugl.: Berlin, Freie Univ., Diss., 2014
Dissertation, Freie Universität Berlin
D 188

Choriner Str. 85 - 10119 Berlin
verlag@menschundbuch.de – www.menschundbuch.de

Dedicated to

my grandma and my grandpa

Contents

List of Abbreviations

ACD	**A**llergic **C**ontact **D**ermatitis
AD	**A**topic **D**ermatitis
AHAPS	*N*-(6-**a**mino**h**exyl)-**a**mino**p**ropyltrimethoxy**s**ilane
APC	**A**ntigen-**p**resenting **C**ell
APS	(3-**a**mino**p**ropyl-)trimethoxy**s**ilane
B cells	**B** lymphocytes
DAB	3,3'-**d**i**a**mino**b**enzidine
DAPI	4′,6-**d**i**a**midino-2-**p**heny**l**indole
DC	**D**endritic **C**ell
DNA	**D**eoxyribo**n**ucleic **A**cid
DLS	**D**ynamic **L**ight **S**cattering
ELISA	**E**nzyme-**l**inked **I**mmuno**s**orbent **A**ssay
FITC	**F**luorescein-5-**i**so**t**hio**c**yanate
HaCaT	Immortal human keratinocyte cell line
HE	**H**ematoxylin and **E**osin
HEL-30	Murine keratinocyte cell line
HeLa	Human epithelial cervical carcinoma cell line originated from the female patient **He**nritta **La**cks
Hr	**H**ai**r**less
IFN	**I**nter**f**ero**n**
Ig	**I**mmuno**g**lobulin
IL	**I**nter**l**eukin

i.v.	**i**ntra**v**enous
LC	**L**angerhans **C**ell
MHC	**M**ajor **H**istocompatibility **C**omplex
NM	**N**ano**m**aterial
NP	**N**ano**p**articles
FLIM	**F**luorescence **L**ifetime **I**maging **M**icroscopy
HPF	**H**igh-**P**ower **F**ield
Ova	**Ova**lbumin
Ox	**Ox**azolone
PBS	**P**hosphate **B**uffered **S**aline
PEG	**P**oly**e**thylene **G**lycol
QD	**Q**uantum **D**ots
RAW264.7	Murine macrophage cell line
RLN	**R**egional **L**ymph **N**ode
ROS	**R**eactive **O**xygen **S**pecies
s.c.	**S**ub**c**utaneous
SC	**S**tratum **C**orneum
SEM	**S**tandard **E**rror of the **M**ean
SiO_2-NP	**Si**lica **N**ano**p**articles
T cells	**T** lymphocytes
TEM	**T**ransmission **E**lectron **M**icroscopy
TEWL	**T**rans**e**pidermal **W**ater **L**oss
TGF	**T**ransforming **G**rowth **F**actor
TiO_2-NP	**Ti**tanium **Dioxide N**ano**p**articles

TLR-1	Murine hepatocyte cell line
TNF	**T**umor **N**ecrosis **F**actor
UV light	**U**ltra **V**iolet Light
ZnO-NP	**Zi**n**c** **Oxide** **N**ano**p**articles

1 Introduction

Nanoparticles (NP) are defined as material with a particle size less than 100 nm in at least one dimension. On the one hand, NP occur in nature in dust or as remnants of combustion processes. On the other hand, engineered NP are produced in a controlled way for several purposes. In contrast to their bulk material, NP possess distinctly different characteristics and, thus, NP are used in a variety of new applications. For example, they can be found as compounds of cosmetics, clothes, food as well as in electronics, paints and polishes, amongst others, because they hold valuable optical, tactile or even antibacterial properties (Buzea et al. 2007; Wiesenthal et al. 2011). In recent years, various nanomaterials (NM) have been exploited for biomedical applications, such as gene or drug delivery systems as well as for medical imaging purposes (Salata 2004). During particle synthesis the physicochemical properties, such as shape, size and surface charge, can be adjusted easily (Roduner 2006). The use of NP as drug delivery systems, for example, is of prime interest because with NP the enclosure and controlled release of substances at specific target tissues becomes possible. This application reduces the required amount of a certain drug and thereby also possible side effects (Slowing et al. 2008). One promising representative of NP in novel biomedical applications are silica NP (SiO_2-NP; Nabeshi et al. 2011b).

The new characteristics of NP provide many benefits but they may also entail several risks. Thus, the toxicity of NP has to be evaluated for the risk assessment of new NP species. As NP and viruses are of similar size (Buzea et al. 2007), it is likely that NP can overcome selected barriers of the body, such as skin, lung or intestine, and get access to the body. The skin as the largest organ of the body with up to 10 % of the body mass is an important interface between the organism and NP regardless of their intended or unintended exposure (Crosera et al. 2009). The penetration of NP through healthy skin has been discussed controversially in recent years, depending on the type and size of the NP, the vehicle and model used (Labouta and Schneider 2013). Furthermore, the role of a disturbed skin barrier on the penetration of NP remains to be elucidated, although some authors have suggested that particle penetration might be favored (Prow et al. 2011). Superficial skin barrier disruptions and skin diseases, such as allergic contact dermatitis (ACD), are very common in humans with a prevalence of ACD of about 20 % (Thyssen et al. 2007). The widely spread use of NP in biomedical as well as commercial applications, i.e., cosmetics or sunscreens, increases the risk of exposure of NP to healthy and diseased skin. However, data on the penetration behavior in such conditions are insufficient (Prow et al. 2011).

Moreover, the skin provides innate as well as acquired immune response functions. As suggested by several studies on murine allergic disease models, NP may have an immunomodulatory effect (DeLouise 2012). To date, in the majority of models treatment of such animals with NP and an allergen concurrently resulted in disease aggravation. So far, the role of NP exposure during an allergic disease remains unclear, regardless of the allergen contact.

This project aimed at identifying the effect of different skin barrier disruptions on the local penetration and systemic distribution of SiO_2-NP in mouse models. Furthermore, the course and outcome of SiO_2-NP exposure during an already existing allergic contact dermatitis in a mouse model were studied. The results are expected to add more information for the development of SiO_2-NP-based approaches in biomedical sciences and contribute to the risk assessment of such NP.

1.1 Nanomaterial and Nanoparticles

Nanomaterials (NM) are defined as materials with a particle size between 1 and 100 nm in at least one dimension including nanoparticles (NP) as well as nanofilms and nanocomposites (Sayes and Warheit 2009). The Greek word “nanos”, meaning “dwarf”, is the origin of the prefix “nano” (Buzea et al. 2007).

NP can be either of natural origin or man-made. Natural occurring NP, for example, are particles produced by microorganisms such as magnetotactic bacteria synthesizing magnetite NP. However, NP can be found in aerosols as part of dust and erosion, volcanic eruptions or forest fires. Up to 90 % of aerosols are of natural origin and affect the air quality in several regions worldwide with related health problems such as asthma or cardiovascular diseases. Man-made NP are either unintentionally made NP, for example, particles of vehicle exhaust, or they might be engineered intentionally for their favored characteristics. However, NP show unique properties compared to larger scaled or bulk materials. This is caused by the higher surface area of NP per mass unit. For example, the surface area-to-volume ratio for 60 nm NP is 1,000 times larger than for a 60 µm particle (Buzea et al. 2007). Additionally, each NP is composed of a cluster of atoms. The atoms at the NP surface have less direct neighbors than the atoms of bulk materials with the consequence of a decreased binding energy per atom in NP. This results in a reduced melting point for smaller sized particles. Furthermore, when NP are thermally excited, they may become magnetic although their bulk material does not show magnetic properties. Moreover, the so-called quantum effects are accountable for tunable optical properties such as emission of fluorescent signals at certain wavelengths. This quantum effect is determined by the standing waves of a confined system and not due to the

fraction of surface atoms. In conclusion, the physicochemical properties can be aligned in a very large range by changing the size of NP (Roduner 2006). By modifying the physico-chemical properties, the interaction of NP with biological systems are also different compared to their corresponding bulk material (Lin et al. 2006; Napierska et al. 2010; Rancan et al. 2012). This is mainly explained by the altered surface area per mass unit (Colvin 2003; Oberdörster et al. 2005). Having this knowledge, advancements in various fields including biomedicine become now possible and will be discussed later.

NP can be grouped based on their primarily chemical composition into organic and inorganic NP (Papakostas et al. 2011). Organic NP are usually soft particles that are made of organic elements, such as carbon, oxygen, hydrogen and their polymers (Papakostas et al. 2011; Sayes and Warheit 2009). The inorganic NP are generally composed of a variety of inorganic materials, including metal, metal oxides as well as ceramics. Typically, inorganic NP are solid particles (Papakostas et al. 2011). Due to the plethora of different organic and inorganic NP species and the use of inorganic silica NP in this study, only inorganic NP will be covered and discussed in the following.

Nanotechnology as one of the leading technologies in the twenty-first century involves the engineering and chemistry of NP for novel applications (Hubbs et al. 2013; Zhao et al. 2011). The expectations for new applications of NP are tremendous (Zhao et al. 2011). In biomedicine, NP are used as drug or gene delivery systems, fluorescent biological label or contrast agents in diagnostic imaging, tissue engineering and *in vivo* targeting of cancer cells. Moreover, NP even possess adjuvant properties (Prow et al. 2011; Salata 2004). However, the interaction of NP with cells of a living organism is a critical process. On the one hand, NP should enter cells to execute their function *in vivo*. On the other hand, this cellular uptake raises concerns on potential risks, meaning toxic effects, of NP applications (Zhao et al. 2011). In light of the expected worldwide distribution of NP and the likelihood of human exposure regardless of intentional or unintentional exposure, the safety evaluation of NP should be of prime interest (Oberdörster et al. 2005). Human health might be affected by inhalation, ingestion and / or dermal contact of NM (Park et al. 2010). Several human diseases are known for long time to be induced by NP, such as asbestos inducing lung fibrosis, lung cancer and mesothelioma or quartz (e.g., crystalline silica) inducing a lung disease called silicosis (Donaldson and Seaton 2012). But not only the inhalation of NP is associated with a health risk, a condition called podoconiosis, in which people develop a lymphoedema in the limbs, is thought to arise from the dermal uptake of NP by barefoot walking on soil (Buzea et al. 2007). Furthermore, an increased release of engineered NP for certain purposes into the environment can be assumed from their extensive use (Graf et al. 2012). Thus, the risk of human exposure to NP is more than likely. As a consequence, the discipline of nanotoxicology has emerged.

The effects of mainly engineered NP exposure to living organisms are the main focus of this discipline (Oberdörster et al. 2005).

1.2 Pathways of Nanoparticle Entry into the Body

As explained earlier, human health may be affected by, e.g., inhalation, ingestion and / or dermal contact of NM (Park et al. 2010). In recent years, the respiratory tract has been in the focus of *in vivo* nanotoxicity studies due to the assumption that airborne NP cause significant health effects (Oberdörster et al. 2005). The inhalation of particles is, in general, related with an accidental inhalation of NP in workplaces and the environment (Donaldson and Seaton 2012). Due to their small size, NP may reach the deep respiratory tract in a considerable high percentage with up to 50 % of inhaled particles. NP might cross epithelial cells to enter the systemic circulation or be taken up by alveolar macrophages (Oberdörster et al. 2005).

So far, only few studies dealt with the fate of ingested NP in the gastrointestinal tract. Reported results are a rapid passage and elimination of NP (Oberdörster et al. 2005).

The skin was for long time considered less permeable (Crosera et al. 2009) and not in the focus of research activities. However, due to its exposed site the skin is a potential uptake pathway and skin toxicity is an important issue (Oberdörster et al. 2005; Park et al. 2010). This will be discussed in the following chapter.

1.3 The Skin in the Focus of this Work

Due to their location, skin cells have the highest frequency of both intentional as well as unintentional NP contact (Nabeshi et al. 2011c; Rancan et al. 2012) and potential skin toxicity of NP is important aspect (Oberdörster et al. 2005; Park et al. 2010). Moreover, the skin offers a painless and favorable interface for systemic drug administration. The transdermal application of substances is a convenient method to reduce the first-pass degradation process in the liver and can even reduce the side effects of the substances (Prausnitz et al. 2004). In addition to medical approaches, a lot of cosmetics contain NP due to their favorable optical and tactile properties (Wiesenthal et al. 2011), and get, consequently, in contact with the skin.

For a better understanding of differences between human and mouse skin as favored animal model, the comparative skin anatomy and physiology between these two species will be explained in the next chapter.

1.3.1 Skin Structure and Physiology

The skin is the largest organ of the body with up to 10 % of the body mass (Crosera et al. 2009). It protects the living organism against desiccation, mechanical, thermal, chemical and biological influences (Liebich 2004). However, the skin can generally be divided into two distinct parts: epidermis and dermis that are separated by the basement membrane.

The epidermis (Figure 1) is the most outer layer of the skin and exhibits the major barrier function (Liebich 2004). The epidermis of mice is with 29 µm thinner compared to 47 µm in humans (Godin and Touitou 2007). The epidermis consists to more than 90 % of keratinocytes (Merad et al. 2008). Within the epidermis, four strata are distinguished on the basis of the degree of differentiation. The *stratum basale* is composed of the most immature keratinocytes that adhere to the basement membrane by hemidesmosomes. Keratinocytes are interconnected with each other via desmosomes. The basal cells proliferate and as the differentiation continues, keratinocytes become more flatten in the *stratum spinosum*. Usually, the spinous layer of mice has a thickness one cell layer and, thus, is much thinner than human skin. Cells become more elongated in the *stratum granulosum* and secrete complex lipids from their lamellar bodies. The outermost layer, the *stratum corneum* (SC), is composed of cornified non-nucleated keratinocytes (Treuting and Dintzis 2011). The murine SC has a thickness of approximately 9 µm, whereas the human SC is about 17 µm in size (Godin and Touitou 2007). The SC provides the main barrier function of the skin (Breternitz et al. 2007). The cells of the SC are arranged like the “bricks in a wall” that are held together by the secreted lipids that act as “mortar” (Elias 1983). The lipids of the SC consist mainly of ceramides, cholesterol and free fatty acids that are arranged into multi-lamellar bilayers (Choi et al. 2003; Prausnitz et al. 2004).

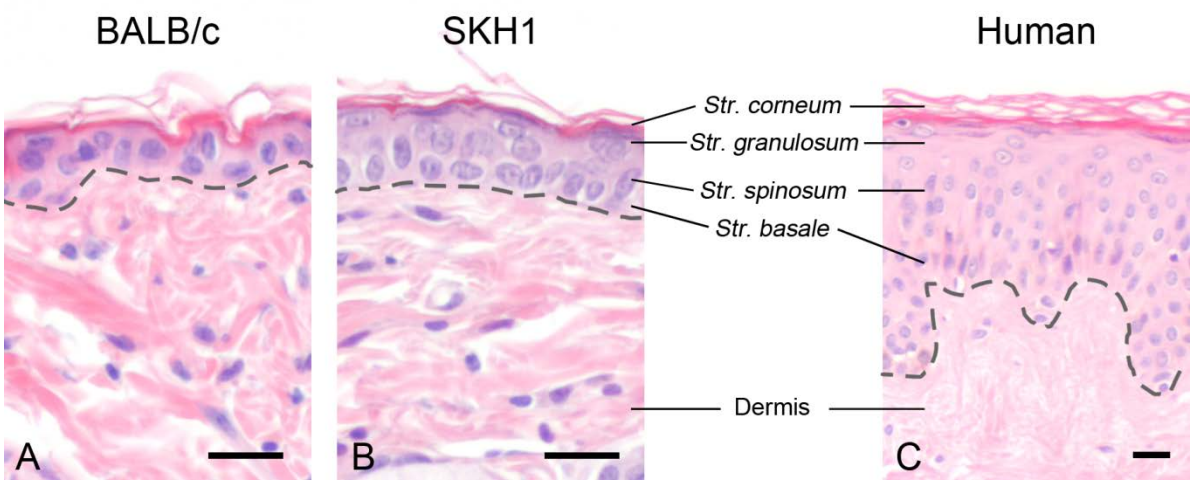

Figure 1: Comparative skin histology of BALB/c mice, SKH1 mice and humans (here shown without hair follicles). The epidermis of mice with complete fur (A) is thinner compared to the epidermis seen in hairless SKH1 mice (B). However, humans (C) have a significantly thicker

epidermis compared with both mouse strains. The gray dashed line represents the basement membrane. Hematoxylin and eosin (HE); *Str. corneum* = *stratum corneum; Str. granulosum* = *stratum granulosum; Str. spinosum* = *stratum spinosum; Str. basale* = *stratum basale*. Bars = 20 µm. Source: Anja Ostrowski

The dendritic cells (DC) of the epidermis are called Langerhans cells (LC) and represent between 3 and 5 % of nucleated epidermal cells in mice and humans (Merad et al. 2008). The LC extend their dendrites between the keratinocyte tight junctions without disturbing the skin barrier (Kubo et al. 2009). These antigen-presenting cells (APC) are during resting state in close contact with the keratinocytes via E-cadherin-mediated adhesion and start their trans-migration to the regional lymph node (RLN) as early as 16 hours following mechanical stress (Kissenpfennig et al. 2005).

The dermis is composed of a meshwork of fibroblasts, elastic fibers, type I and III collagens as well as extracellular matrix molecules including proteoglycans and glycoproteins (Treuting and Dintzis 2011). It contains blood vessels and a variety of immune cells, including mast cells, memory T lymphocytes (T cells) and dermal DC (Kaplan 2010).

The pilosebaceous unit in mice consists of the hair follicle, associated sebaceous glands and the arrector pili muscle (Treuting and Dintzis 2011). The basal membrane accompanies the epithelial root sheath of hair follicle into the depth of the dermis (Liebich 2004). However, due to the hairless phenotype of SKH1 mice, the pilosebaceous unit is more or less negligible in this study. Besides the pilosebaceous units, eccrine sweat glands show a bodywide distribution in humans, whereas in mice, they can only be found on the foot pads (Treuting and Dintzis 2011).

1.3.2 Barrier Function of the Skin

The skin protects the living organism from several external influences (Liebich 2004) and comprises several types of barriers. The physical barrier consists primarily of the SC as explained above and the apical intercellular spaces between the viable cells of the epidermis are sealed with tight junctions (Proksch and Brasch 2012; Rancan et al. 2012). The (bio)chemical barrier acts as innate immunity and is provided by lipids, acids, lysozymes and antimicrobial peptides (Proksch and Brasch 2012). The redox barrier is provided by the sulfur-rich layer of the SC implementing a biochemical buffering function (Pickard et al. 2009). The immune barrier, i.e., cellular and humeral immunity, provides protection against infectious diseases but may overreact in allergic diseases (Proksch and Brasch 2012). These barriers do not work independently, rather they work together. For example, it has been shown that a physical skin

barrier disruption or an inflammatory skin disease leads to an increase in antimicrobial peptides (Ahrens et al. 2011; Glaser et al. 2008). Thus, the skin barrier is a complex network in which the SC is of prime interest due to its main physical barrier function.

1.3.3 Testing of Nanoparticles in *in vivo* Skin Models

In order to test new substances in terms of penetration and skin toxicity *in vivo* animal models are still indispensable although the most reliable data can only be collected in human studies. Despite the fact that porcine skin resembles the human skin in the best way, skins of rodents, such as mice, are commonly used as animal model due to their small size, uncomplicated handling and low costs (Godin and Touitou 2007). In particular, the skin of hairless animals mimics the human skin quite well due to the absence of a dense hair coat. Thus, no clipping or shaving is necessary and skin injuries are avoided (Godin and Touitou 2007; Simon and Maibach 1998).

In this study, the hairless SKH1 mouse was used. These mice are suitable for dermatology research as well as safety and efficacy testing (Charles River Laboratories International 2014). In contrast to fully haired mice, the SKH1 mouse has a thicker epidermis and, thus, their skin mimics the skin of humans more closely (Blomme et al. 2003). Moreover, SKH1 mice are an euthymic outbred strain of hairless mice with an autosomal recessive mutation termed *hr* in the *hairless* (*Hr*) gene (Benavides et al. 2009; Schaffer et al. 2010). The mutation of the *Hr* gene results in the failure to regrowth hairs after the first shedding (Potter et al. 2001). The expression of *Hr* in progenitor keratinocytes is indispensable for normal hair growth. With increasing age of SKH1 mice, the rugosity of the skin proceeds, hair follicle cysts may develop, sebaceous glands enlarge and culminate in the development of dermal granulomas (Benavides et al. 2009). In contrast to homozygous nude mice, the SKH1 mouse strain has a comparable immune competence to the widely used C57BL/6 mouse strain (Schaffer et al. 2010).

1.3.4 Pathways of Skin Penetration

As explained earlier, animal models are commonly used for testing the penetration behavior of new substances. However, the murine skin is more prone to the penetration of substances through the skin especially due to its thinner epidermis in comparison to the human epidermis (Godin and Touitou 2007). Hence, the use of hairless mouse skin in penetration studies erred on the side of safety, i.e., the penetration through murine skin is greater than through human skin (Simon and Maibach 1998).

In general, three penetration pathways for substances through the skin are described (Figure 2): (1) transcellular, (2) intercellular and (3) appendageal (transfollicular) route, i.e., along the hair follicles and eccrine glands (Hadgraft 2001). The latter two ones are the main skin penetration routes for NP (Labouta and Schneider 2013).

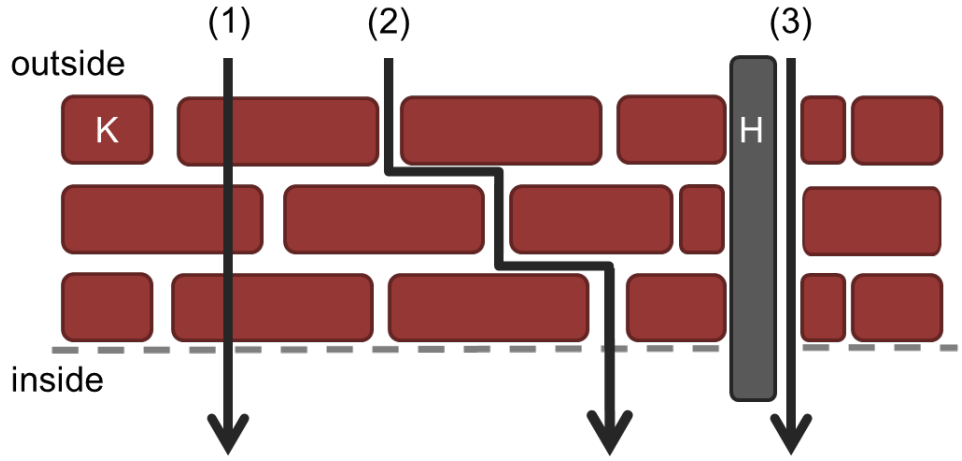

Figure 2: Possible pathways of penetration through the skin (modified from Hadgraft, 2001): The black arrows demonstrate possible paths through the epidermis (K, keratinocytes; H, hair follicle); (1): transcellular pathway, (2): paracellular pathway, (3): transfollicular pathway. The gray dashed line represents the basement membrane.

The paracellular pathway is the passive diffusion along a tortuous route within the lipids of the SC (Prausnitz et al. 2004). Several authors have described a NP penetration via the hair follicles due to the large opening within the epidermis (Baroli 2010; Lademann et al. 2009a; Lademann et al. 2009b). The hair follicle might act as long-term reservoir (Lademann et al. 2009b; Lademann et al. 2007). The moving hair pushes the NP into the follicle by acting as pumping system *in vivo* (Labouta and Schneider 2013; Lademann et al. 2009a). Moreover, LC concentrate in the epithelium of hair follicle infundibulum (Vogt et al. 2006). Despite this fact, the transfollicular pathway plays only a minor role in the hairless mouse used in our study.

1.4 Penetration of Inorganic Nanoparticles Through Intact Skin

As explained above, the skin is an important organ for a complete risk assessment of NP exposure. An intact skin barrier is an outstanding protection against several environmental influences. However, the skin barrier is compromised under diseased conditions, such as

mechanical superficial skin injuries or in inflammatory skin conditions. It was thus assumed that an impaired skin barrier leads to an enhanced particle penetration (Prow et al. 2011).

As a first step in studies on NP penetration, the integrity of the skin barrier should be assessed *in vivo* by measuring the transepidermal water loss (TEWL). Thus, TEWL reflects the status of the skin barrier. As a general assumption, it is believed that the degree of barrier disturbance correlates with drug penetration in accordance with an “outside-in” concept (Fluhr et al. 2006; Proksch and Brasch 2012). The TEWL can be assessed by various commercially available devices all measuring the water evaporation from the epidermis. The measurement with an open-chamber system, for example, Tewameter TM 300, is based upon diffusion principles in an open cylinder system with a defined surface area that is covered by the probe. Two sensors within the probe detect the evaporated water depending on time. In SKH1 mice, values below 10 g/m^2h are described as intact skin (Fluhr et al. 2006).

Although the physicochemical properties of NP play a crucial role in the penetration of the skin, characterizing all factors influencing NP skin penetration is still difficult. Often the modification of a certain parameter involves the modification of other factors as well (Labouta and Schneider 2013). However, a size-dependent penetration has been reported for gold NP and titanium dioxide NP (TiO_2-NP) (Labouta et al. 2011; Sonavane et al. 2008; Wu et al. 2009). Gold NP are often used as model NP to study skin penetration of NP but they are also a promising canditate in drug delivery or as tool in thermal tumor destruction (Labouta et al. 2011). TiO_2-NP are of high interest in their skin penetration as they are a major ingredient of sunscreens due to their ability to scatter, absorb and reflect ultra violet (UV) light (Papakostas et al. 2011). In conclusion, an increased penetration should be expected with decreasing particle size (Baroli 2010). The shape may also influence penetration behavior, so it has been shown for various shaped quantum dots (QD) that spherical QD penetrate faster than ellipsoid QD. The interest in QD is substained to their great potential for use as diagnostic and imaging agents (Ryman-Rasmussen et al. 2006). These QD typically consist of a semiconductor core (e.g., cadmium sulfide), an outer shell of a higher band semiconducting material (e.g., zinc sulfate) and a surface functionalization which may consist of various hydrophilic organic molecules including biomolecules or polymers (Medintz et al. 2005). QD have size-dependent optical properties (Roduner 2006) and posses a superior photostability, a high quantum yield as well as a broad excitation and narrow emission spectra (Mortensen et al. 2008) that even allow for the detection of mixed QD populations with a single excitatory wavelength (Medintz et al. 2005). However, QD release toxic ions on biological matter. This can been minimized by embedding QD into silica nanoparticles (Graf et al. 2006) without influencing their optical properties (Gerion et al. 2001).

Solvents, surfactant or chemical enhancers that are used as disolution vehicle for NP may alter or temporarily damage the SC and thereby enhance penetration of NP (Baroli 2010; Labouta and Schneider 2013). The choice of the vehicle should also be made with respect to the colloidal stability of the NP. This is of prime interest because a reduced colloidal stability would result in an aggregation of NP (Knopp et al. 2009) with the consequence of altered NP properties (Graf et al. 2012) and each change in the physicochemical properties of NP may have tremendously different biological tolerability. NP aggregation might occur in different vehicles due to the ionic strength of different vehicles (Graf et al. 2012). It has been discussed for gold NP that single NP have a higher probability to penetrate as it can be expected for aggregated NP (Labouta et al. 2011). Other factors influencing the penetration are NP concentration and exposure time of the NP (Labouta and Schneider 2013).

The literature concerning the penetration of inorganic NP is partially contradictory. For example, several authors reported a penetration of QD with different size, shape and surface coatings through intact murine skin *in vivo* and porcine skin in an *in vitro* / *ex vivo* setup (Chu et al. 2007; Mortensen et al. 2008; Nabeshi et al. 2011b; Ryman-Rasmussen et al. 2006). In contrast, other studies using similar QD and experimental designs revealed no penetration of QD through intact human, rat and mouse skin beyond the SC *in vitro* and *in vivo* (Gopee et al. 2009; Gratieri et al. 2010; Jeong et al. 2010; Zhang and Monteiro-Riviere 2008; Zhang et al. 2008). The same is true for TiO_2-NP. In the majority of studies, TiO_2-NP failed to reach viable epidermal layers but they were detected in hair follicles in *in vivo* setups using rat, human and pig skin as well as in *in vitro* setups using human and pig skin (Adachi et al. 2010; Gontier et al. 2008; Lademann et al. 1999; Mavon et al. 2007; Newman et al. 2009; Sadrieh et al. 2010; Senzui et al. 2010). In contrast, Wu and colleagues failed to detect TiO_2-NP beyond the SC following *in vitro* exposure to porcine skin but following a 30-day-treatment of pigs *in vivo*, only the smallest TiO_2-NP with a diameter of 4 nm were located in viable epidermal layer and induced mild pathological changes. Furthermore, a 60-day-treatment of mice resulted in a systemic distribution of TiO_2-NP and pathological changes were observed in the skin, liver, spleen and lung (Wu et al. 2009). For zinc oxide NP (ZnO-NP), also an important ingredient of sunscreen similar to TiO_2-NP, and silver NP, that are favorable due to antiseptic properties, it has been suggested that there is only minimal penetration through intact human skin *in vitro* (Cross et al. 2007; Larese et al. 2009). Using excised rat skin, a penetration of variously sized gold NP has been reported. In that study, 15 nm gold NP penetrated the skin faster and in a higher amount compared to larger sized gold NP with a diameter of 102 nm or 198 nm, respectively (Sonavane et al. 2008). Other small sized metallic NP, for example, maghemite and iron NP, have been shown to penetrate into hair follicles and the SC, and to occasionally reach viable epidermal layers (Baroli et al. 2007).

Taken together, drawing general conclusions on possible particle penetration through intact skin by knowing the physicochemical characteristics is impossible. It seems that an intact skin barrier provides a good protection shield against particle penetration, although all experimental setups have to be taken into account. However, contradictory results are also obtained even when similar setups were used. So each study has to be evaluated carefully and should give as much experimental details as possible, for example, NP size, shape and charge as well as the vehicle and detection methods used.

1.5 Mechanical Skin Barrier Disruptions

Skin barrier disruptions are quite common. It can be assumed that a compromised skin barrier leads to enhanced particle penetration (Prow et al. 2011). In addtion to chemical enhancers that temporarily reduce the skin barrier function, various physical methods have been described to either study the determinants of NP penetration or to reinforce penetration of substances and NP (Labouta and Schneider 2013; Prausnitz et al. 2004). Apart from mechanical injuries, the integrity of the skin barrier is also compromised in diseased skin, which is discussed in the next chapter.

1.5.1 Models of Mechanical Skin Barrier Disruption

In the literature, several models of mechanical, i.e., physical, skin barrier disruptions have been described to study the role of the skin barrier in NP penetration or even to enhance the penetration of NP through the skin. It can be assumed that in general each of the methods described in the following leads to a damage of the skin barrier, which is mainly provided by the SC.

Slight skin injuries can occur following physical forces (Senzui et al. 2010) as they occur after scratching, for example (Ilves et al. 2014). For this purpose, tape stripping is an inexpensive and minimally invasive method to disrupt the skin barrier by removing the SC. Tape stripping is described in various species, including humans and mice (Gopee et al. 2009; Mavon et al. 2007). Adhesive films are pressed onto the surface of the skin and then removed together with the superficial layer of the SC (Breternitz et al. 2007). It is commonly used to study the SC or to enhance drug penetration. Tape stripping results in the production and release of interleukins (IL) 1α, 1β, 8 and 10, tumor necrosis factor (TNF) α and Interferon (IFN) γ. The expression of co-stimulatory molecules and the capacity of antigen-presenting DC are enhanced (Choi et al. 2003) and leads, as well as the application of chemical irritant, to an activation of LC. 16 hours following a mechanical trauma LC start to become motile and emigrate to the RLN where DC were found in substantial numbers as early as 24 hours after stress induction

(Kissenpfennig et al. 2005). This migration is induced by the cytokines TNF α and IL 1β (Valladeau and Saeland 2005). Furthermore, it has been shown that following tape stripping the density of Langerhans cells in the epidermis increases (Nishijima et al. 1997; Proksch and Brasch 1997).

Cyanoacrylate stripping, which is commonly used for human skin samples, disrupts the skin barrier and opens the hair follicles for particle entry (Schaefer and Lademann 2001; Vogt et al. 2006). This method has been described for human and porcine skin (Senzui et al. 2010; Vogt et al. 2006) but cannot be used in mice. In human skin samples, one cyanoacrylate stripping removes about 30 % of the SC and leaves the remaining SC and deeper epidermal layers intact (Vogt et al. 2006). A single cyanoacrylate stripping is used in excised human skin to model the *in vivo* situation of intact human skin samples due to the reduced skin elasticity after excision. However, the intercorneocyte junctions may be damaged slightly and consequently, cyanoacrylate stripping does not exactly model the *in vivo* situation of healthy skin (Rancan et al. 2012).

An increased damage of the skin barrier is achieved by dermabrasion where the epidermis is removed by a Dremel or aluminium oxide microcrystals (Gopee et al. 2009; Lee et al. 2006). Dermabrasion is a commonly used technique for skin resurfacing or drug delivery (Grimes 2005; Orentreich and Orentreich 1995). It has been described in murine and porcine animal models as well as in humans (Gopee et al. 2009; Grimes 2005; Lee et al. 2006).

Exposure of the skin to UV light leads to a skin barrier disruption by disorganization of intercellular lipid lamellae (Jiang et al. 2006), and thereby impairs the inside-out-barrier function as assessed by TEWL (Mortensen et al. 2008). This method can be used in animal models including mice and pigs (Monteiro-Riviere et al. 2011; Mortensen et al. 2008). The relevance of UV light damaged skin is explained by the use of TiO_2-NP and ZnO-NP as common protecting ingredient of sunscreens (Labouta and Schneider 2013).

The integrity of the skin barrier can also be disrupted mechanically by flexing and massaging the skin (Labouta and Schneider 2013). Flexing of excised skin with a flexing apparatus should simulate the mechanical stress as it is seen in flexing movement *in vivo* (Zhang and Monteiro-Riviere 2008). However, this approach has only been described in excised rat skin and, thus, it is not clinically acceptable. To reach the acceptability, massaging of the skin might be preferred and more applicable (Labouta and Schneider 2013), especially in *in vivo* setups. The massage should mimic the rubbing movements during the application of a substance *in vivo* (Gratieri et al. 2010). During massaging, the hairs are moved and so the hair cuticles have been suggested to act as geared pump that works in *in vitro* and *in vivo* setups (Lademann et al. 2007). This method has been applied so far in excised human and porcine skin as well as

in human *in vivo* skin studies (Gratieri et al. 2010; Lademann et al. 2007). Furthermore, a skin massage is likely to increase local temperature by enhanced vascularization and favor the ingress of NP *in vivo* (Baroli 2010).

Moreover, microneedles have been used for transdermal drug delivery by creating micron sized holes in the epidermis without inducing pain or significant damage (Prausnitz et al. 2004). This methods has been used so far *in vitro* for human skin and *in vivo* for rat skin (McAllister et al. 2003). Other physical enhancement methods, for example, with the help of an electric field (iontophoresis and electroporation) or by using ultrasound to move molecules (sonophoresis), will only be mentioned and have not been used extensively in penetration studies of NP (Labouta and Schneider 2013; Prausnitz et al. 2004).

1.5.2 Literature Review on the Penetration of Inorganic Nanoparticles Through a Mechanically Disrupted Skin Barrier

Similar as for intact skin, contradictory results concerning the penetration of various inorganic NP through a disrupted skin barrier have been reported. Gopee et al. reported an *in vivo* penetration of 37 nm sized QD only in dermabraded skin of mice where the complete epidermis has been removed. However, they failed to detect a penetration following tape stripping and acetone pretreatment, respectively (Gopee et al. 2009). Similar results were reported for QD studied in excised rat skin. The QD penetration was limited to the SC in intact, flexed and tape stripped skin but a dermabrasion resulted in dermal penetration in that study (Zhang and Monteiro-Riviere 2008). Using excised human skin, a penetration of QD in deeper epidermal layers was only observed after a combination of tape stripping and massaging for ten minutes (Gratieri et al. 2010). The use of mild local hyperemia in mice resulted in an increased skin penetration of QD *in vivo* as well (Upadhyay 2006). Different sized gold NP were able to penetrate to deeper skin layers in excised human skin following tape stripping using different vehicles (Labouta et al. 2011). The group of Larese demonstrated an increased penetration of silver NP following dermabrasion (Larese et al. 2009). It has been shown for TiO_2-NP that even following tape stripping no penetration was observed in excised porcine skin, but a removal of hairs resulted in an increased epidermal concentration of TiO_2-NP (Senzui et al. 2010)

One group showed an uptake of 40 nm, but not of 750 nm or 1500 nm polystyrene NP by LC following topical application of cyanoacrylate stripped, excised human skin (Vogt et al. 2006). Polystyrene NP are often used as model particle to study NP cell interactions (Varela et al. 2012).

Following UV light pretreatment of murine skin *in vivo* an increased penetration of 20 nm QD into deeper epidermal layers and the dermis has been reported (Mortensen et al. 2008). A

deeper epidermal, but no dermal penetration of 50 nm TiO_2-NP and 140 nm ZnO-NP was reported in an *in vivo* porcine model with UV light skin treatment prior particle application (Monteiro-Riviere et al. 2011).

In summary, the different experimental designs, different NP species and different vehicles used in all studies do not allow for a general conclusion on which disruption method model is the most appropriate one to study the *in vivo* situation in humans and what are the main factors influencing the penetration of NP.

1.6 Barrier Disruptions in Inflammatory Skin Diseases

1.6.1 General Aspects

Apart from mechanical injuries, the integrity of the skin barrier is also impaired in inflamed skin. In inflammatory skin diseases, such as allergic contact dermatitis (ACD) or atopic dermatitis (AD), the skin barrier is compromised, and this may result in an increased skin penetration of NP (Crosera et al. 2009). These barrier disruptions might be explained by modifications in epidermal thickness, metabolic capacity and microstructure of SC as well as the disruption of the surface integrity in lesional skin (Baroli 2010).

Substances intended for application in diseased skin are generally studied with intact skin, neglecting differences in the altered environment. Those application include therapeutic purposes, such as NP for drug delivery, or preventive purposes, for example, NP in sun-screens. However, not much is known about the penetration of NP in inflamed skin so far (Baroli 2010).

In addition to this, NP may act as adjuvant by enhancing immune reactivity or modulate the immune response (DeLouise 2012; Prow et al. 2011). The skin is the main route for allergen sensitization, and NP themselves may have negative immunological effects following intentional or unintentional exposure (DeLouise 2012).

1.6.2 Allergic Contact Dermatitis in Humans

ACD is a common skin disorder in European and North American people with a prevalence of approximately 20 %. It is induced by skin contact with a hapten that exceeds the individual threshold (Thyssen et al. 2007). Haptens are electrophilic molecules with low molecular weight that are able to penetrate the skin. Due to their small size, haptens are not immunogenic themselves but bind covalently to cutaneous proteins and form an immunogenic hapten-protein complex (Christensen and Haase 2012). As brief outline of the pathogenesis, ACD represents the skin reaction resulting from exposure to a hapten or allergen and can be divided

into two different phases, the sensitization and elicitation phase (Christensen and Haase 2012; Proksch and Brasch 2012). Proksch and Brasch reviewed the ACD and described an enhanced epidermal proliferation as well as an allergen-specific T cells initiated inflammatory skin reaction (Proksch and Brasch 2012). For a more detailed description on the pathogenesis of ACD, see the chapter of animal model on ACD. Finally, ACD results in a significantly impaired barrier function and, consequently, additional sensitization may evolve after contact with other allergens (Proksch and Brasch 2012).

Patients suffering from ACD are usually treated in first line with corticosteroids (Schlapbach and Simon 2014). However, corticosteroids and other treatments can be used only for a limited time period due to side effects (Proksch and Brasch 2012). Thus, using NP as drug delivery system to reduce the side effects is of prime interest.

1.6.3 Animal Models of Allergic Contact Dermatitis

Several animal models of ACD and AD have been described. Mouse models of ACD are one of the best studied *in vivo* models of immune activation and are usually induced with haptens, such as oxazolone (Ox).

The first contact between the skin and the hapten initiates the sensitization phase. The formed hapten-protein complexes are taken up by APC (Christensen and Haase 2012). The production of TNF α and IL 1β by keratinocytes and LC leads to migration of APC to the RLN and subsequent T cell activation in the RLN (Christensen and Haase 2012; Cumberbatch et al. 1997). Ox activates both IFN γ and IL 4-synthesizing T cells (Christensen and Haase 2012). The subsequent exposure of the hapten used for sensitization initiates the elicitation phase. T cell recruitment due to vasoactive and inflammatory mediators, such as TNF α leads to emigration of specific T cells to the site of elicitation, but B lymphocytes (B cells) and natural killer T cells seemed to be also involved in this phase (Christensen and Haase 2012). However, the immunologic response following sensitization with Ox is not clearly defined as T helper (Th) 1 or Th2 type. The sensitization with Ox results in increased serum levels of immunoglobulin E (IgE) (Christensen and Haase 2012). It has been suggested that besides Th1 also Th2 cytokines play an important role during the elicitation phase of ACD (Schlapbach and Simon 2014). The resulting significant disturbance of the skin barrier supports additional sensitization, which might evolve after contact with other allergens (Proksch and Brasch 2012).

1.6.4 Atopic Dermatitis in Humans

AD is a chronic relapsing itchy inflammatory skin disease with an IgE-involvement that impairs the quality of life of affected people. The prevalence of AD is between 15 and 30 % in children

and up to 10 % in adults with an upward trend in the last decades (Bieber 2010). AD-patients are susceptible to develop contact dermatitis and show a xerosis, i.e., dry skin, in lesional and non-lesional areas. Similar as in ACD, the skin barrier of AD-skin is also impaired and the histologic pictures of both diseases are identical (Bieber 2010; Proksch and Brasch 2012). The pathomechanism includes a genetic predisposition, among others a mutation in the filaggrin gene has been identified. Filaggrin is a key protein in the terminal differentiation of the epidermis (Bieber 2010), and its mutation results in a defective skin barrier function (Schlapbach and Simon 2014).

In addition to decreased expression of antimicrobial peptides, AD-patients show a systemic Th2-dominated dysbalance with increased IgE levels and eosinophilia. Despite the Th2-deviated inflammation, the AD skin lesions are biphasic. In the acute phase, elevated levels of Th2 cytokines, IL 4, 5 and 13 are detectable in lesional and non-lesional skin areas. These cytokines are responsible for initial inflammation, increased expression of adhesion molecules in endothelial cells and increased eosinophil survival. In the chronic phase, Th1 cytokines, including IFN γ, IL 11, 12 and 18 as well as transforming growth factor (TGF) β 1, seem to be responsible for apoptosis and remodeling (Bieber 2010).

The management of AD is in first terms the topical use of emollients to support the barrier function and / or anti-inflammatory agents, such as corticosteroids or Tacrolimus, to reduce exaggerated immune response (Bieber 2010; Schlapbach and Simon 2014). This approach is sufficient in most cases, but in more severe cases a systemic treatment with immunosuppressant drugs, such as cyclosporine, might be needed (Schlapbach and Simon 2014). However, so far no satisfactory therapy is available for the treatment of AD (Jin et al. 2009).

1.6.5 Animal Models of Atopic Dermatitis

Several mouse models for AD have been described. They can be grouped in one of three categories: (1) treatment-induced, (2) transgenic mice with overexpression or lack of certain molecules and (3) spontaneous development of AD in certain mouse strains (Jin et al. 2009). Due to the poor to fair reproducibility of the two latter categories (Shiohara et al. 2004), only the induced models will be explained in more detail in the following. In the first group, AD is induced by applying sensitizer. Those sensitizers can be allergenic proteins, such as ovalabumin (OVA) or house dust mite allergen, or haptens, as described for ACD, only with prolonged repetitive treatments during the elicitation phase (Jin et al. 2009). In addition to a topical treatment, the proteins can also be injected intradermally to induce the AD-models (Hirai et al. 2012b; Hirai et al. 2011; Hirai et al. 2012c).

1.6.6 Literature Review on the Performance of Inorganic Nanoparticles in Inflammatory Skin Diseases

To date, there is a lack of knowledge regarding the capacity of NP to penetrate diseased skin as well as their capacity to provoke and / or promote allergic diseases. NP may act as adjuvants and, thus, the development of allergic diseases might be initiated or promoted by NP. Allergic diseases seem to be facilitated by NP via IgE-dependent response and / or interactions with immune cells (Shannahan and Brown 2014).

There are only two studies focusing on the penetration of NP through diseased skin with contradictory results. One study revealed that there is no penetration of 35 nm ZnO-NP across the SC of human volunteers with AD (Lin et al. 2011). In contrast to these results, Ilves and colleagues reported a penetration of 20 nm ZnO-NP into deeper epidermal and even dermal layers using an AD mouse model. But 240 nm ZnO-Np were only localized on the skin surface in that study (Ilves et al. 2014). Furthermore, an accumulation of ZnO-NP in the hair follicles was reported. The authors reasoned that inflamed skin might result in a higher particle penetration, at least for 20 nm ZnO-NP. However, the sensitized skin was tape stripped before particle application simulating scratching behavior of patients. Despite the location of 20 nm ZnO-NP in viable skin layers, the authors reported an alleviation of AD lesions by reduced cellular influx of inflammatory cells and skin thickness (Ilves et al. 2014).

Regarding a possible interaction with the immune system, it has been shown that following intradermal injection of polystyrene NP or TiO_2-NP together with dust mite antigen an aggravation of AD-like skin lesions in mice was observed. A size-dependent aggravation with smallest NP initiating the greatest effects was only seen for polystyrene NP but not for TiO_2-NP (Yanagisawa et al. 2009; Yanagisawa et al. 2010).

Compared to the penetration of NP trough intact skin, there is a lack of knowledge on NP penetration and their performance in such diseased skin conditions. The pathomechanisms remain elusive and more research has to be done on this field for complete risk assessment of certain NP species intended for dermal applications.

1.7 Silica Nanoparticles

1.7.1 Silica Nanoparticles and their Biomedical Applications

Silicon dioxide, also known as silica (SiO_2), is a major natural component of sand and quartz and, thus, one of the most abundant materials on earth (Jaganathan and Godin 2012). However, in recent years, engineered silica NP (SiO_2-NP) are produced on a large scale as additive

to drugs, food and even printer toner as well as varnishes (Manh Quan et al. 2010; Morimoto et al. 2013; Nabeshi et al. 2011c). These NP can be produced in a controlled way and one can customize their characteristics for application-specific needs (Knopp et al. 2009). SiO_2 is known to be biocompatible and its use is considered to be safe for humans (Barik et al. 2008; Luo and Saltzman 2005). The cosmetic industry uses the colorless SiO_2-NP because they reflect and diffract UV light more efficiently than particles in the micrometer range (Nabeshi et al. 2011c; Wang et al. 2003). In the field of biomedicine, SiO_2-NP have been developed, for example, in cancer therapy, gene and drug delivery system (Napierska et al. 2010). The tunable properties of SiO_2-NP and their relevance for biomedical applications will be explained in the following.

SiO_2-NP can be prepared inexpensively with variable size and shape and subsequently be easily modified (Graf et al. 2012; Yoshida et al. 2012). In contrast to SiO_2 in micrometer scale, the smaller sized SiO_2-NP can be engulfed more efficiently by mammalian cells which is crucial for biomedical applications, such as drug delivery into certain cells (Slowing et al. 2008). Different synthesis protocols may result in different biological behavior of SiO_2-NP due to the different NP properties resulting from each method. It has been shown that the porosity and the surface characteristics had the highest impact on the toxicity *in vitro* and *in vivo* (Yu et al. 2012a; Yu et al. 2011). Mesoporous SiO_2-NP have a honeycomb-like structure and are useful for drug delivery. The porous structure allows for the absorption of quite large amounts of bioactive molecules and their controlled release becomes possible (Slowing et al. 2008). However, Yu and colleagues showed that increased particle porosity resulted in an increased toxicity (Yu et al. 2012a; Yu et al. 2011). This was explained by a higher protein absorption to mesoporous SiO_2-NP compared to nonporous SiO_2-NP (Yu et al. 2012a). The most common method to synthesize SiO_2-NP is the so-called Stöber synthesis (Knopp et al. 2009). Using this method, nonporous particles are built that have a smaller surface area compared to mesoporous SiO_2-NP (Yu et al. 2012a). Furthermore, the particle size can be tuned finely using Stöber synthesis. The size of NP is of particular interest as it was shown that smaller sized NP (< 60 nm) exhibit a higher tumor penetration and retention than larger NP (Tang et al. 2013). In addition to this and as explained earlier, with decreasing particle size the surface area per mass unit increases resulting in a higher chemical activity (Buzea et al. 2007). Smaller sized NP are more likely engulfed by various mammalian cells than particles in the micrometer range (Slowing et al. 2008). For visualization purposes, SiO_2-NP may be covalently labeled with a fluorescent dye, for example, fluorescein-5-isothiocyanate (FITC) (Graf et al. 2012; Knopp et al. 2009; van Blaaderen et al. 1992; van Blaaderen and Vrij 1992). It has been shown that an incorporation of a fluorescent dye into the core of SiO_2-NP increased the photostability of the

certain dye and enables their use as imaging tool for *in vivo* visualization of capillaries, sentinel lymph nodes and tumor metastasis (Choi et al. 2007; Helle et al. 2013; Jeon et al. 2010).

Unfunctionalized SiO_2-NP have a negative zeta potential, meaning surface charge, due to silanolate groups on the surface of the NP (Graf et al. 2012). These silanolate groups on the surface of pure SiO_2-NP allow for functionalization with different groups such as amine, carboxy, aldehyde and other cross-linkers (Knopp et al. 2009). The different functionalizations may have a tremendous effect on the physicochemical properties as well as on their performance in biological systems (Graf et al. 2012). By modifying the surface with *N*-(6-aminohexyl)-aminopropyltrimethoxysilane (AHAPS), the negatively charged, unfunctionalized SiO_2-NP become positively charged. Furthermore, AHAPS-functionalization results in an increased colloidal stability in biological media and an increased cellular uptake of SiO_2-NP *in vitro* (Graf et al. 2012). The colloidal stability is of prime interest as explained earlier. Besides this, AHAPS enables the binding of deoxyribonucleic acid (DNA) to the particles and even prevents the enzymatic degradation of the DNA (Kneuer et al. 2000b) transforming SiO_2-NP to a promising gene delivery system (Luo and Saltzman 2005). This has successfully been proven *in vitro* and *in vivo* (Kneuer et al. 2000a; Kumar et al. 2004).

Taken together, nonporous SiO_2-NP possess enormous potential in the development of several new biomedical applications, especially due to their high biocompatibility.

1.7.2 Biological Behavior of Silica Nanoparticles *in vitro*

Because of its size and exposed site, the skin is an important route of NP entry into the body (Oberdörster et al. 2005). As the epidermis consists of 90 % of keratinocytes (Merad et al. 2008), many groups used keratinocyte cell lines to study possible cytotoxic effects of SiO_2-NP. Nabeshi and colleagues have shown a nuclear location of 70 nm SiO_2-NP and growth inhibition of human keratinocyte cell line (HaCaT) following incubation with 70 nm and 300 nm SiO_2-NP. Furthermore, a size-dependent mutagenicity has been shown, i.e., the smaller the SiO_2-NP the more serious the effects (Nabeshi et al. 2011b). Membrane damage has only been observed for 70 nm SiO_2-NP in dose-dependent manner, but not for 300 nm and 1000 nm particles (Nabeshi et al. 2011c). Furthermore, the exposure of SiO_2-NP exerted toxic effects and altered protein expression in HaCaT cells. The altered proteins included, among others proteins associated with oxidative stress, metabolism, apoptosis and tumor-associated proteins (Yang et al. 2010). Another group also using HaCaT cells demonstrated a reduced viability following exposure to 7 nm and 10 - 20 nm SiO_2-NP but cytotoxic effects were seen between the two different sizes used. Furthermore, no irritation potential was observed in human skin equivalent model in that study (Park et al. 2010). However, in murine keratinocytes (HEL-30), various sized SiO_2-NP with a diameter between 30 nm and 535 nm were found in

intracytoplasmic vacuoles and endosomes but not in the nucleus. In that study, membrane leakage increased with decreased SiO_2-NP size and 30 nm and 48 nm SiO_2-NP showed a significant cytotoxicity but no increase in reactive oxygen species (ROS) generation was observed (Yu et al. 2009).

Using different cell lines, the cytotoxic effects of SiO_2-NP were studied to evaluate their presumed *in vivo* performance. A size-dependent uptake and intracellular location of SiO_2-NP has been described. 300 nm and 1000 nm SiO_2-NP were located in the cytoplasm of a murine LC line, whereas 70 nm SiO_2-NP were localized in the cytoplasm and nucleus of these cells. However, cellular damage of LC was induced by 70 nm and 300 nm SiO_2-NP in a dose-dependent manner (Nabeshi et al. 2010). In addition to the particle size, surface functionalization might play an important role in the biological behavior of NP by changed NP properties. Some groups have shown an increased cellular uptake by HaCaT and the human epithelial cervical carcinoma cell line (HeLa) cells following a surface functionalization resulting in a positive surface charge compared to unfunctionalized and therefore negatively charged SiO_2-NP (Graf et al. 2012; Rancan et al. 2012). This phenomenon was explained by an increased interaction of positively charged SiO_2-NP with negatively charged cell membrane (Graf et al. 2012). In addition to this, it seems that the surface functionalization correlates with cytotoxic effects of NP. A functionalization with amine or carboxyl groups resulted in a reduced cytotoxicity in different cell lines, among others HaCaT cells, murine hepatocytes (TLR-1) and murine macrophages (RAW264.7), compared to unfunctionalized SiO_2-NP (Morishige et al. 2012; Nabeshi et al. 2011a; Yoshida et al. 2012). Only unfunctionalized SiO_2-NP but no amine- or carboxyl-functionalized SiO_2-NP have been found to enter the nucleus of RAW264.7 cells (Nabeshi et al. 2011a).

Intracellular ROS might be induced by NP exposure via metabolic processes or activation of oxygen. Reactive nucleophilic molecules of oxygen, i.e., superoxide anion, are built due to these primary ROS with a consequent interaction with other molecules, such as enzymes, which in turn results in the promotion of secondary ROS that mediate DNA damage (Nabeshi et al. 2011c). Oxidative DNA damage is related to carcinogenesis and mutagenesis (Ames 1983). It has been shown that SiO_2-NP induced ROS generation in various human cell lines (Lin et al. 2006; Liu and Sun 2010; Nabeshi et al. 2011c). It seems that a surface functionalization of 70 nm SiO_2-NP with amine or carboxyl groups results in a reduced ROS generation and DNA damage in HaCaT cells and a murine hepatocyte cell line (Yoshida et al. 2012).

Moreover, the uptake capacity of various cell types is different. Thus, it has been shown that primary LC incorporate more SiO_2-NP than primary keratinocytes. Furthermore, the uptake

mechanisms and competence varies between immortalized cell lines, such as HaCaT cells and primary keratinocyte cell culture (Rancan et al. 2012).

In conclusion, the biological behavior of SiO_2-NP depends on physicochemical properties, such as size and surface functionalization of the NP. Despite this assumption, the literature on biological effects is partially contradictory due to different SiO_2-NP, different surface functionalizations and different cell lines used in those studies.

1.7.3 Biological Behavior of Silica Nanoparticles *in vivo*

In order to evaluate the possible risks of NP exposure, *in vivo* studies are required in addition to *in vitro* studies. The complexity of a living organism cannot be modeled in a culture dish so far. Thus, animal models are employed to study the *in vivo* effects of NP. However, the toxicological response is often inconsistent (Nabeshi et al. 2011a). On the one hand, SiO_2 is believed to be a non-toxic material (Kumar et al. 2004). Several studies revealed no systemic toxicity of SiO_2-NP in various experimental setups *in vivo* (Fu et al. 2013; Helle et al. 2013). On the other hand, some authors showed toxic effects of SiO_2-NP exposure to biological systems (Nabeshi et al. 2011b; Nishimori et al. 2009b). Yu and colleagues stated that the *in vivo* toxicity of SiO_2-NP was mainly influenced by NP surface characteristics and porosity (Yu et al. 2012a).

Following intravenous (i.v.) injection of various sized SiO_2-NP the NP were localized in the liver, mainly in Kupffer cells and to a lesser extent in hepatocytes, and might be excreted in the bile. They were also found in the lung, kidney, spleen and lymph nodes in smaller amounts (Kumar et al. 2010; Nabeshi et al. 2011b). The NP showed a cytoplasmic and nuclear location (Nabeshi et al. 2011b). In general, i.v. injection of SiO_2-NP leads to an uptake of NP by the cells of the reticulo-endothelial system following sequestration in the liver and spleen. Furthermore, it seems that surface characteristics and porosity may play a notable role in the biodistribution of SiO_2-NP (Yu et al. 2012b). A systematic study of different exposure routes revealed a low absorption of SiO_2-NP in the liver following subcutaneous (s.c.) injection, whereas the i.v. injection leads to an accumulation in liver and spleen (Fu et al. 2013). The hepatobilary and urinary routes are favored for excretion of SiO_2-NP (Cho et al. 2009; Fu et al. 2013; Helle et al. 2013; Kumar et al. 2010; Nabeshi et al. 2011b).

In several studies, the authors reported a size-dependent SiO_2-NP induced hepatotoxicity whereas kidney, lung, heart and brain remained unaffected. The smaller particles induced a tissue damage even in low doses whereas even high doses of bigger sized SiO_2-NP had no effects. Furthermore, prolonged i.v. exposure to SiO_2-NP resulted in a hepatic fibrosis (Nishimori et al. 2009a, 2009b). This hepatotoxicity seemed to occur due to cytotoxic effects

and DNA damage induced by 70 nm SiO_2-NP (Nabeshi et al. 2011b). Moreover, i.v. application of SiO_2-NP resulted in a consumptive coagulopathy and pregnancy complications (Nabeshi et al. 2012; Yamashita et al. 2011). In contrast to this, others had not seen any adverse effects following i.v. injection of organically modified SiO_2-NP with a diameter of about 25 nm (Kumar et al. 2010). In addition, in one study, the authors reported a SiO_2-NP induced inflammatory response for 100 nm and 200 nm NP, but i.v. injection of 50 nm NP was not associated with such response (Cho et al. 2009).

Only few reports can be found in the literature which focused on the skin toxicity of SiO_2-NP *in vivo*. A topical exposure to 70 nm sized, unfunctionalized SiO_2-NP seemed to induce a cellular damage in the mouse skin (Nabeshi et al. 2011b), whereas the group of Park did not see any acute irritation potential of 7 nm and 10 - 20 nm SiO_2-NP in the rabbit skin model (Park et al. 2010).

In summary, not only *in vitro* studies but also *in vivo* studies on SiO_2-NP application revealed partially contradictory results for their systemic tolerability. Due to the different experimental setups concerning SiO_2-NP themselves, the vehicle used, application route and frequency as well as the NP dose no generalized conclusions can be drawn.

1.7.4 Penetration of Silica Nanoparticles Through Intact Skin

In the literature, only few studies investigated the dermal penetration of SiO_2-NP in intact skin. It has been demonstrated that a topical application of unfunctionalized SiO_2-NP with size of 70 nm to mice resulted in skin penetration and systemic distribution after a three-day- and 28-day-treatment period *in vivo* (Hirai et al. 2012a; Nabeshi et al. 2011b). Following a three-day-treatment, 70 nm SiO_2-NP were found in epidermal LC, the dermis and even in the RLN (Hirai et al. 2012a). After 28 days of treatment, SiO_2-NP in 10 % isopropyl myristate, a well-known solubilizer, were localized in the skin, the RLN, hepatocytes and even in the brain. Additionally, an increase in apoptotic cells in the skin of particle-treated animals compared to the water control has been reported. The systemic distribution and the pro-apoptotic effects were only seen for 70 nm SiO_2-NP but not for 300 and 1000 nm SiO_2-NP (Nabeshi et al. 2011b). An *in vitro* study on SiO_2-NP that were coated with the lipophilic fluorescent label acridine orange 10-nonyl-bromide and porcine skin revealed a penetration of NP into viable epidermal layers (Eskandar et al. 2010).

At least two of the three studies cited here, used a lipophilic environment for particle application and, thus, these lipophilicity might likely interact with the lipids of the SC. Furthermore, it remains questionable if water-treated animals were the appropriate control to validate the vehicle effects of isopropyl myristate used by Nabeshi and colleagues. Although SiO_2-NP are

used frequently in cosmetics and their potential for future applications as transdermal drug delivery tool, the knowledge on the SiO_2-NP penetration through intact skin is insufficient. Hence, further studies are necessary for a better understanding on the penetration and a complete risk assessment of SiO_2-NP.

1.7.5 Penetration of Silica Nanoparticles Through a Mechanically Disrupted Skin Barrier

Only two studies made a mechanical skin barrier disruption a subject on their research. Following a single cyanoacrylate stripping, Graf et al. localized 161 nm SiO_2-NP with a gold core in the superficial layers of the SC and in the epithelium of superficial parts of hair follicles in excised human skin (Graf et al. 2009). Similar results were obtained for spherical SiO_2-NP with diameter between 42 nm and 291 nm also in excised human skin (Rancan et al. 2012). However, the authors of that study reported a cellular association of small amounts of 42 nm SiO_2-NP with epidermal cells and LC without differences between unfunctionalized and (3-aminopropyl-)trimethoxysilane (APS)-functionalized NP. This observation was explained by a direct NP contact with the viable epidermis following the cyanoacrylate stripping (Rancan et al. 2012).

So far, no *in vivo* studies focused on the penetration of SiO_2-NP through a mechanically disrupted skin barrier, despite the use of SiO_2-NP in cosmetics and the high incidence of skin barrier disruptions.

1.7.6 Performance of Silica Nanoparticle Exposure in Inflammatory Skin Diseases

Nowadays no data on the penetration of SiO_2-NP through inflamed skin as seen in ACD have been published. However, there are some reports suggesting an immunotoxicity of SiO_2-NP. The group of Hirai and colleagues demonstrated an increased inflammatory response following SiO_2-NP exposure in a murine AD model (Hirai et al. 2012b; Hirai et al. 2011; Hirai et al. 2012c). Both the intradermal injection and the topical application of SiO_2-NP together with the dust mite antigen and Ova, respectively, resulted in a size-dependent exacerbation of the AD-like skin lesions with most deleterious effects for the smallest SiO_2-NP with a particle diameter of 70 nm (Hirai et al. 2012b; Hirai et al. 2011; Hirai et al. 2012c).

However, the role and fate of SiO_2-NP exposure in an already existing ACD is still unclear and remains elusive. Especially for potential drug delivery purposes, it is likely that SiO_2-NP will be used as topical treatment in an already diseased inflammatory environment.

1.8 Aims and Hypotheses of this Study

Any new NP species intended for future application with exposure to humans or animals have to be evaluated concerning their risk and toxicity prior to their use and exposure to living organisms. Several studies have proposed an increased penetration of NP through the skin when the skin barrier is disrupted, such as in inflammatory skin diseases. However, due to partially contradictory results, the role of skin barrier disruption on the penetration and subsequent systemic distribution on NP remains elusive. As shown for unfunctionalized SiO_2-NP, a penetration through intact skin barrier and subsequent systemic distribution seemed possible (Nabeshi et al. 2011b). In order to assess potential hazards regarding the exposure of AHAPS-functionalized SiO_2-NP to the skin, two aims and hypotheses guided this research project:

The **first aim** was to identify the effect of different skin barrier disruptions on the local penetration and systemic distribution of AHAPS-functionalized SiO_2-NP in the mouse. The comparison of the previous studies is difficult due to the vast diversity in experimental designs. **The hypothesis was that a disruption of the skin barrier leads to enhanced local penetration and subsequent systemic distribution of AHAPS-functionalized silica nanoparticles.** Models of mechanical and inflammatory skin barrier disruptions were studied in comparison to intact skin regarding the local penetration of AHAPS-functionalized SiO_2-NP following their topical application. Furthermore, the systemic distributions of SiO_2-NP after topical treatment and s.c. injection were examined using fluorescence microscopy and transmission electron microscopy (TEM). Light microscopy and TEM were also used for the assessment of possible adverse effects.

The **second aim** was to characterize the course and outcome of a model of allergic contact dermatitis following exposure to AHAPS-functionalized SiO_2-NP. **The hypothesis was that AHAPS-functionalized silica nanoparticles aggravate allergic contact dermatitis in the murine model.** Several studies had shown an exacerbation of allergic skin diseases when NP were simultaneously applied with the allergens. Of note, the consequences of an exposure of NP in general and AHAPS-SiO_2-NP in particular during an already existing ACD have not been studied so far. Thus, to evaluate the course and outcome of oxazolone-induced ACD, clinical, histological and immunohistochemical parameters as well as serum IgE contents were compared between normal and diseased skin or animals, respectively.

2 Own Research Publications in Scientific Journals

2.1 Skin Barrier Disruptions in Tape Stripped and Allergic Dermatitis Models Have No Effect on Dermal Penetration and Systemic Distribution of AHAPS-Functionalized Silica Nanoparticles

Authors: Ostrowski A, Nordmeyer D, Boreham A, Brodwolf R, Mundhenk L, Fluhr JW, Lademann J, Graf C, Rühl E, Alexiev U, Gruber AD

Year: 2014

Journal: Nanomedicine: Nanotechnology, Biology, and Medicine

Bibliographic Source: *Ostrowski A, Nordmeyer D, Boreham A, Brodwolf R, Mundhenk L, Fluhr JW, Lademann J, Graf C, Rühl E, Alexiev U, Gruber AD (2014) Skin barrier disruptions in tape stripped and allergic dermatitis models have no effect on dermal penetration and systemic distribution of AHAPS-functionalized silica nanoparticles. Nanomedicine: Nanotechnology, Biology, and Medicine 2014, 10:1571-1581, DOI: 10.1016/j.nano.2014.04.004*

Declaration of own portion of work in this research publication:

Contributions of A Ostrowski:

1. Drafting and development of the study design including animal test proposal, preparation, conduct and evaluation of all experiments involving animal experiments, histology, immunohistochemistry, fluorescence microscopy and transmission electron microscopy

2. Subsequent setup of the entire manuscript with the exception of investigations involving particle synthesis and characterization as well as fluorescence lifetime imaging microscopy

Contributions of the other authors: Participation in the development of study design, evaluation of experimental results and the creation and review of the manuscript, preparation, conduct and evaluation of investigations involving nanoparticle preparation and characterization and fluorescence lifetime imaging microscopy; Subsequent compilation of parts of the manuscript relating to these analyses

Declaration on ethics:

All animal procedures were approved by the Ethics Committee of the local governmental authorities (Landesamt für Gesundheit und Soziales Berlin, approval ID G 0209/11) and were conducted in strict accordance with the Federation of European Laboratory Animal Science Associations (FELASA) guidelines and recommendations for the care and use of laboratory animals (Guillen 2012).

http://www.felasa.eu/recommendations/guidelines/felasa-guidelines-and-recommendations/

DOI: http://dx.doi.org/10.1016/j.nano.2014.04.004

2.2 AHAPS-Functionalized Silica Nanoparticles Do Not Modulate Allergic Contact Dermatitis in Mice

Authors: Ostrowski A, Nordmeyer D, Mundhenk L, Fluhr JW, Lademann J, Graf C, Rühl E, Gruber AD

Year: 2014

Journal: Nanoscale Research Letters

Bibliographic Source: *Ostrowski A, Nordmeyer D, Mundhenk L, Fluhr JW, Lademann J, Graf C, Rühl E, Gruber AD (2014) AHAPS-functionalized silica nanoparticles do not modulate allergic contact dermatitis in mice. Nanoscale Research Letters2014, 9:524, DOI: 10.1186/1556-276X-9-524*

Declaration of own portion of work in this research publication:

Contributions of A Ostrowski:

1. Drafting and development of the study design including animal test proposal, preparation, conduct and evaluation of investigations involving animal experiments, histology, fluorescence microscopy, morphometry and ELISA
2. Subsequent setup of the entire manuscript

Contributions of the other authors: Participation in the development of study design, evaluation of experimental results and the setup and review of the manuscript, preparation, conduct and evaluation of investigations involving nanoparticle preparation and characterization

Declaration on ethics:

All animal procedures were approved by the Ethics Committee of the local governmental authorities (Landesamt für Gesundheit und Soziales Berlin, approval ID G 0209/11) and were conducted in strict accordance with the Federation of European Laboratory Animal Science Associations (FELASA) guidelines and recommendations for the care and use of laboratory animals (Guillen 2012).

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Ostrowski et al. Nanoscale Research Letters 2014, 9:524
http://www.nanoscalereslett.com/content/9/1/524

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AHAPS-functionalized silica nanoparticles do not modulate allergic contact dermatitis in mice

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Abstract

Allergic contact dermatitis (ACD) is a common skin disease in people and may become a potential site of exposure to nanoparticles (NP). Silica nanoparticles (SiO_2-NP) possess a promising potential for various medical and non-medical applications, including normal and diseased skin as target organs. However, it has been shown that negatively charged SiO_2-NP may act as proinflammatory adjuvant in allergic diseases. The effect of topical SiO_2-NP exposure on preexisting ACD has not been studied to date although this reflects a common *in vivo* situation. Of particular interest are the potential effects of positively charged *N*-(6-aminohexyl)-aminopropyltrimethoxysilane (AHAPS)-functionalized SiO_2-NP which are promising candidates for delivery systems, including gene delivery into the skin. Here, the effects of such AHAPS-functionalized SiO_2-NP (55 ± 6 nm in diameter) were studied in an oxazolone-induced ACD model in SKH1 mice and compared to ACD mice treated with vehicle only. The clinical course of the disease was assessed by monitoring of the transepidermal water loss (TEWL) and the erythema. In histologic and morphometric analyses, the distribution of particles, the degree of inflammation, epidermal thickness, and the inflammatory infiltrate were characterized and quantified by standard and special histological stains as well as immunohistochemistry for CD3+ lymphocytes. To assess possible systemic effects, serum immunoglobulin E (IgE) was determined by enzyme-linked immunosorbent assay. Following administration of AHAPS-SiO_2-NP for five consecutive days, no effects were observed in all clinical, histologic, morphometric, and molecular parameters investigated. In conclusion, positively charged AHAPS-SiO_2-NP seem not to affect the course of ACD during exposure for 5 days.

Keywords: Allergic contact dermatitis; Oxazolone; Toxicopathology; Mouse model; Silica nanoparticles

Background

Silica nanoparticles (SiO_2-NP) are among the most promising inorganic nanoparticles (NP) for biomedical applications, such as drug and gene delivery systems, including vaccination [1,2]. In addition, they are used in everyday products like cosmetics [3].

Several studies have shown pro-inflammatory adjuvant effects of SiO_2-NP during ovalbumin or mite antigen-induced allergic dermatitis and allergic airway disease when NP and antigen were administered simultaneously as immunogenic challenge [4-8]. However, no study has addressed possible effects of intended or unintended NP exposure in an already existing allergic contact dermatitis (ACD) which affects approximately 20% of European and North American people [9].

To date, the *in vivo* adjuvant effects of SiO_2-NP in allergic disease models have only been studied for negatively charged SiO_2-NP, including unfunctionalized and polyethylene glycol (PEG)-functionalized SiO_2-NP [4-8]. However, zeta potentials indicative of biologically relevant surface charges have not always been reported in these studies. The surface charge of NP and thus several aspects of their performance in bioenvironments can largely be modified by different functionalizations [10]. For example, surface functionalization of SiO_2-NP may drastically reduce their *in vitro* and *in vivo* cytotoxicity compared to unfunctionalized SiO_2-NP [10,11]. Moreover, positively charged *N*-(6-aminohexyl)-aminopropyltrimethoxysilane (AHAPS)-functionalization results in increased colloidal stability compared to unfunctionalized SiO_2-NP and markedly reduces its tendency towards aggregation [12].

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Accordingly, it appears reasonable to assume that such surface functionalization may also influence NP behavior in diseased tissues, including ACD. Furthermore, AHAPS-functionalization with positive surface charge also allows for additional applications, including DNA binding for gene delivery or vaccination approaches [13]. However, such particles have not been tested in an ACD environment to date.

Consequently, in the present study, we investigated the effects of positively charged AHAPS-SiO_2-NP in a murine model of acute oxazolone-induced ACD [14].

Methods

Particle synthesis and characterization

Spherical SiO_2-NP with a diameter of 55 ± 6 nm were grown in a seeded growth process around a fluorescein-5-isothiocyanate (FITC)-labeled SiO_2-NP core as reported previously [15,16]. Surface functionalization with AHAPS bearing one primary and one secondary amino group per molecule resulted in NP of high colloidal stability [12] and changed the zeta potential of the SiO_2-NP from highly negative −45 ± 4 mV to highly positive values +37 ± 2 mV. Well-dispersed AHAPS-SiO_2-NP were transferred to ultra-pure water (AlleMan Pharma, Pfullingen, Germany) and remained colloidally stable as indicated by transmission electron microscopy (Figure 1) as well as dynamic light scattering (DLS) measurements of hydrodynamic diameter and zeta potential as previously shown [17].

Animals and study protocol

The study protocol was approved by the State Office of Health and Social Affairs, Berlin (LAGeSo G 0209/11) and adhered to national guidelines. ACD was induced in 6- to 8-week-old, male mice of the outbred SKH1 strain (n = 5; Charles River, Germany) using topical application of oxazolone (Ox; Sigma-Aldrich, Steinheim, Germany) in acetone (Biesterfeld Chemiedistribution, Hamburg, Germany). The animals were sensitized with 1.5% Ox in acetone on the right flank. One week later, a first challenge with 0.5% Ox in acetone was applied to the right flank, followed by a second challenge with 0.5% Ox in acetone 2 days later. Each animal was treated on five consecutive days with 50 μL AHAPS-SiO_2-NP (c = 5 g/L) or ultra-pure water, respectively, per day starting 12 h following the first challenge as previously described in [17]. A solvent group (n = 3) was treated with acetone only to control the effects of Ox. Prior to each treatment, the transepidermal water loss (TEWL) was measured to assess the skin barrier disruption with a Tewameter TM 300 (Courage + Khazaka, Cologne, Germany) as described in [18]. Erythema was determined macroscopically and a photograph was taken daily with a digital camera (NEX-3, Sony, Tokyo, Japan). Twenty-four hours after the last treatment, animals were euthanized and tissues were sampled as described in [17].

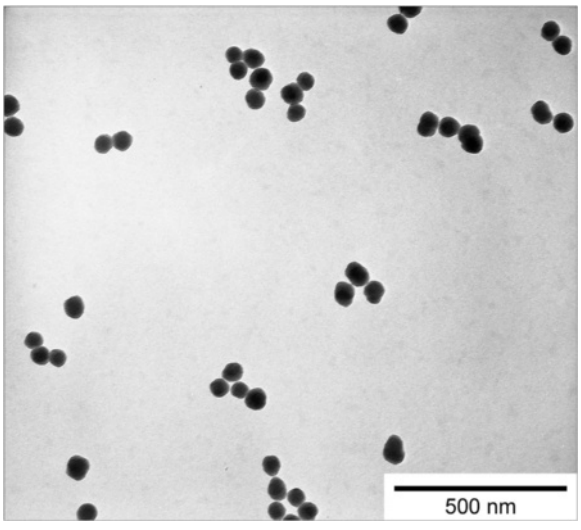

Figure 1 Dispersion characteristics of AHAPS-SiO_2 nanoparticles. Transmission electron microscopy revealed well-dispersed FITC-labeled AHAPS-SiO_2-NP with a diameter of 55 ± 6 nm.

IgE measurements

Blood samples were collected by cardiac puncture immediately following euthanasia and serum immunoglobulin E (IgE) concentrations determined with a mouse IgE enzyme-linked immunosorbent assay (ELISA) quantification kit (Bethyl Laboratories, Montgomery, TX, USA) according to the manufacturer's protocol [19].

Particle localization and histologic and morphometric analyses

Fluorescent AHAPS-SiO_2-NP were localized in 4′,6-diamidino-2-phenylindole (DAPI; Carl Roth, Karlsruhe, Germany) stained section as described in [17]. Furthermore, serial 5-μm sections of formalin-fixed, paraffin-embedded skin were stained following routine protocols with hematoxylin and eosin (HE) and toluidine blue or Congo red for visualization of mast cells or eosinophils, respectively. T lymphocytes were immunohistochemically identified in lesional skin using antibodies to the surface marker CD3 (rabbit polyclonal anti-human CD3, dilution 1:1,500, Dako, Glostrup, Denmark) following heat-induced antigen retrieval with citrate buffer (pH 6.0) as reported in [20].

Samples were examined microscopically using a BX41 microscope (Olympus, Shinjuku-ku, Japan) equipped with a digital camera (Color-View II, SIS, Münster, Germany), a fluorescence burner (U-RLF-T, Olympus, Japan), and a digital image analysis software (AnalySIS docu, version 5.0, SIS, Münster, Germany). Tissues were evaluated

histopathologically and cells were quantified in an observer-blinded manner as described below.

Histologic analysis of lesional skin included the evaluation of hyperkeratosis, parakeratosis, intraepidermal exocytosis, and dermal infiltrate. Epidermal thickness was measured in six randomly selected lesional areas from the basement membrane to the base of the stratum corneum as described in [21]. Mast cells and eosinophils were counted in 20 randomly selected high-power fields (hpf) at × 400 magnification. T lymphocytes were counted in 10 hpf per epidermis and dermis, respectively.

Statistical analyses

Data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed with SPSS software (IBM, Armonk, NY, USA) using one-way ANOVA for normally distributed parameters (IgE, epidermal thickness, and CD3+ cells) or Kruskal-Wallis test for not normally distributed parameters (mast cells and eosinophils). For all tests, significance was considered for $p < 0.05$ with the following designation: $^{*}p < 0.05$ and $^{***}p < 0.001$.

Results

Clinically, the Ox treatment resulted in a marked increase in TEWL values, independently of subsequent treatment with AHAPS-SiO_2-NP or vehicle alone (Figure 2A). The highest values were recorded on treatment day 5 without significant differences between the two treatment groups (AHAPS-SiO_2-NP versus ultra-pure water). Treatment of the skin with acetone alone did not increase TEWL values, and values remained within physiological ranges below 10 g/m^2h [18]. Ox-treated skin but not the skin in the acetone solvent group developed a marked erythema beginning 12 h after the first Ox challenge and lasting for the whole treatment period (Figure 2B,C,D). Consistently, significant higher final serum IgE concentrations were recorded in Ox-treated mice compared to mice from the control group (Figure 3). There were no statistically significant differences between the AHAPS-SiO_2-NP (176.69 ± 36.83 ng/mL) and vehicle (292.14 ± 150.59 ng/mL) treatments.

Using fluorescence microscopy of skin sections, the green fluorescent AHAPS-SiO_2-NP were localized only in the superficial layers of the stratum corneum in all particle-treated mice as shown earlier [17]. No fluorescent signal was detected in the stratum corneum of ultra-pure water-treated animals.

Histologically, the lesional skin of ACD mice showed a mild hyperkeratosis and a severe intraepithelial exocytosis as well as dermal infiltrates. Parakeratosis was minimal in Ox-treated animals. Mice treated with acetone solvent only did not show any histopathological changes. No differences in the histologic parameters were seen between both treatment groups of ACD (Figure 4A,B,C). Morphometrically, the epidermis in Ox-induced ACD was significantly thicker compared to that from mice of the solvent group. In contrast, no significant differences were recorded between epidermal thicknesses in AHAPS-SiO_2-NP-treated versus vehicle-treated ACD mice (Figure 4A,B,

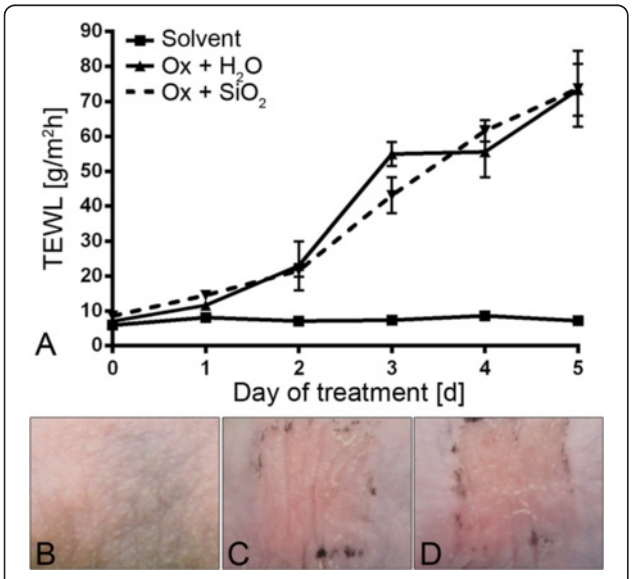

Figure 2 Effects of AHAPS-SiO_2-NP treatments on barrier function and erythema. Transepidermal water loss (TEWL) increased in oxazolone (Ox)-treated mice without significant differences between the AHAPS-SiO_2-NP-treated versus ultra-pure water-treated groups. Solvent-only-treated mice failed to develop elevated TEWL values above the physiological threshold **(A)**. Erythema was absent from the skin of the acetone solvent group **(B)** but present to similar degrees in the vehicle control group **(C)** and AHAPS-SiO_2-NP-treated group **(D)**. Data are presented as mean ± SEM; Ox-treated groups: $n = 5$; solvent-treated group: $n = 3$.

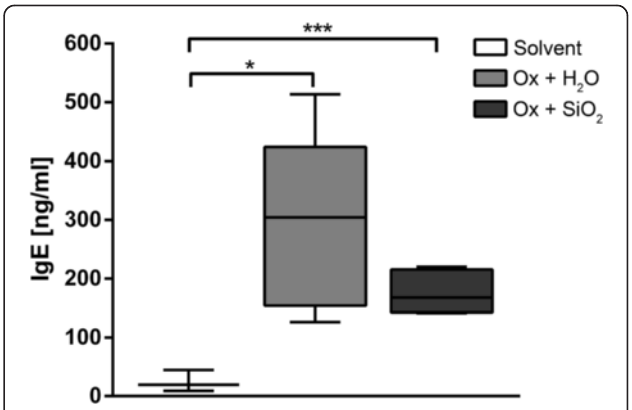

Figure 3 Serum IgE response following topical oxazolone treatment. Serum IgE levels were significantly different between the acetone solvent control and oxazolone (Ox)-treated groups but without significant differences between the vehicle and AHAPS-SiO_2-NP treatments. Data are presented as a box plot with whiskers displaying minimum to maximum values; Ox-treated groups: $n = 5$; solvent-treated group: $n = 3$; $^{*}p < 0.05$, $^{***}p < 0.001$.

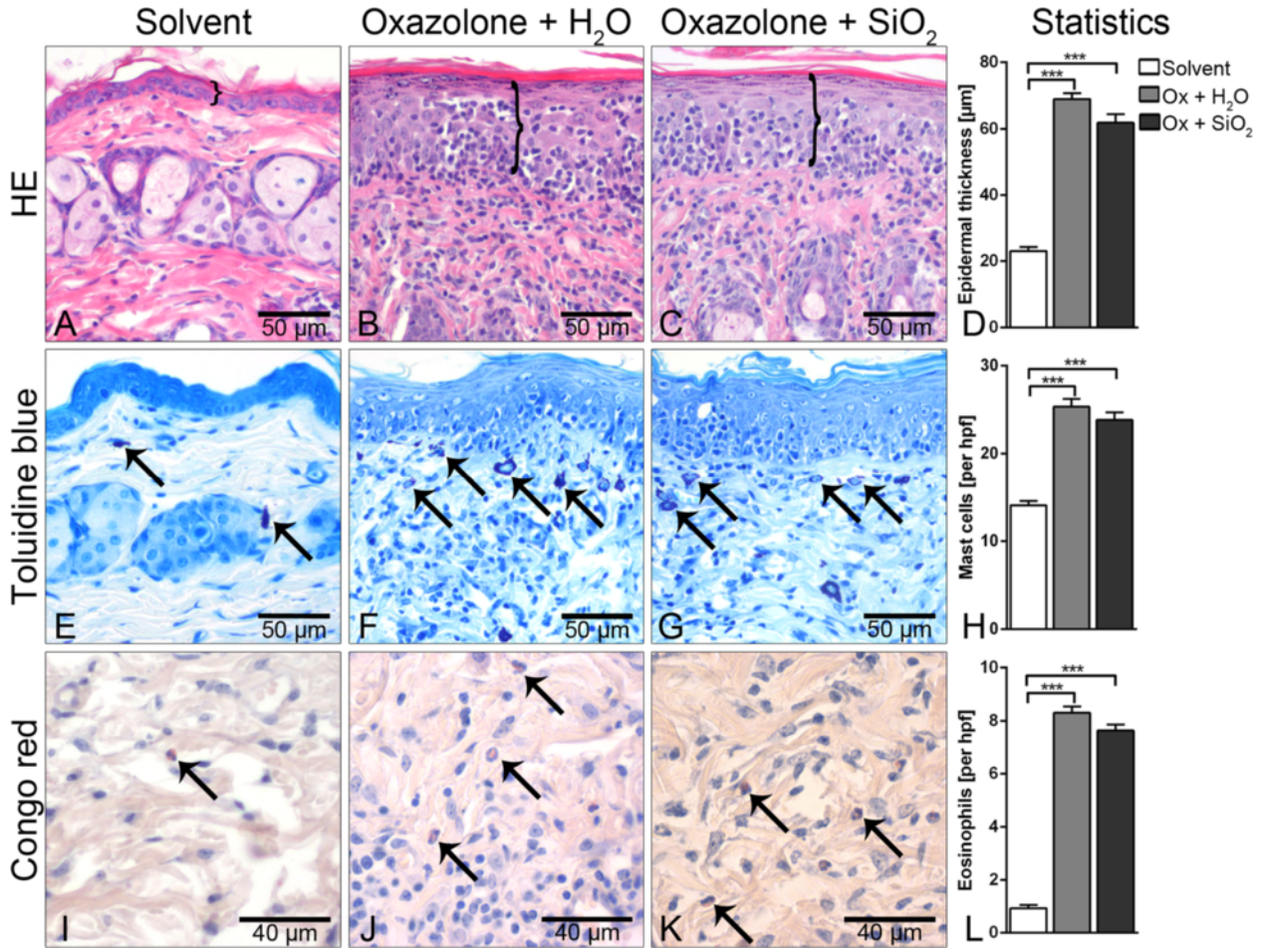

Figure 4 Histologic and morphometric analyses of lesional skin. Hematoxylin and eosin (HE) staining and measurement of epidermal thickness indicated by brackets (top panel) failed to reveal inflammation-associated epidermal thickening in solvent controls **(A)** but showed marked thickening of the epidermis and intraepidermal immune cell exocytosis in oxazolone (Ox)-treated mice without differences between the vehicle control and AHAPS-SiO_2-NP treatments **(B-D)**. Increased infiltrations with mast cells (toluidine blue, central horizontal panel: **E-H**) and eosinophils (Congo red, bottom panel: **I-L**, arrows) were very similar between the two Ox-treated groups. Only a few mast cells and eosinophils were observed in acetone solvent controls **(E, I)**. Images represent typical samples. Quantification included all samples in panels D, H, and L. Data are presented as mean ± SEM; Ox-treated groups: $n = 5$; solvent-treated group: $n = 3$; ***$p < 0.001$.

C,D). Similar results were obtained for the numbers of mast cells and eosinophils (Figure 4E,F,G,H,I,J,K,L) as well as for T lymphocytes infiltrating the epidermis and dermis (Figure 5). The data of each group for all morphometric, histologic, and immunohistochemical parameters are summarized in Table 1.

Discussion

In contrast to previous studies according to which skin barrier disruptions result in increased penetration of irritants with aggravation of inflammatory disease [22], we could not detect any effects of AHAPS-SiO_2-NP on the quality or degree of inflammation. Specifically, our results are at variance with previous studies using negatively charged SiO_2-NP that had aggravated allergic reactions in the skin and airways including the same dose as used here [4-8]. Among possible explanations of this discrepancy is the fact that AHAPS-SiO_2-NP do not penetrate beyond the stratum corneum, the outermost layer of the skin, in normal and irritated skin, including the mice used here. The distribution and levels of penetration of AHAPS-SiO_2-NP on the skin of ACD mice have been studied in detail in a previous report of our group [17]. In that study, no pathological changes were recorded in healthy mice following exposure to AHAPS-SiO_2-NP [17]. Furthermore, NP had been injected intradermally in the majority of previous studies [5,6]. Consequently, in these studies, the SiO_2-NP reach viable epidermal layers and may directly interact with keratinocytes and immune cells. Thus, an injection poorly models the *in vivo* situation in human and murine ACD where NP are topically applied to the skin and have to overcome the relatively

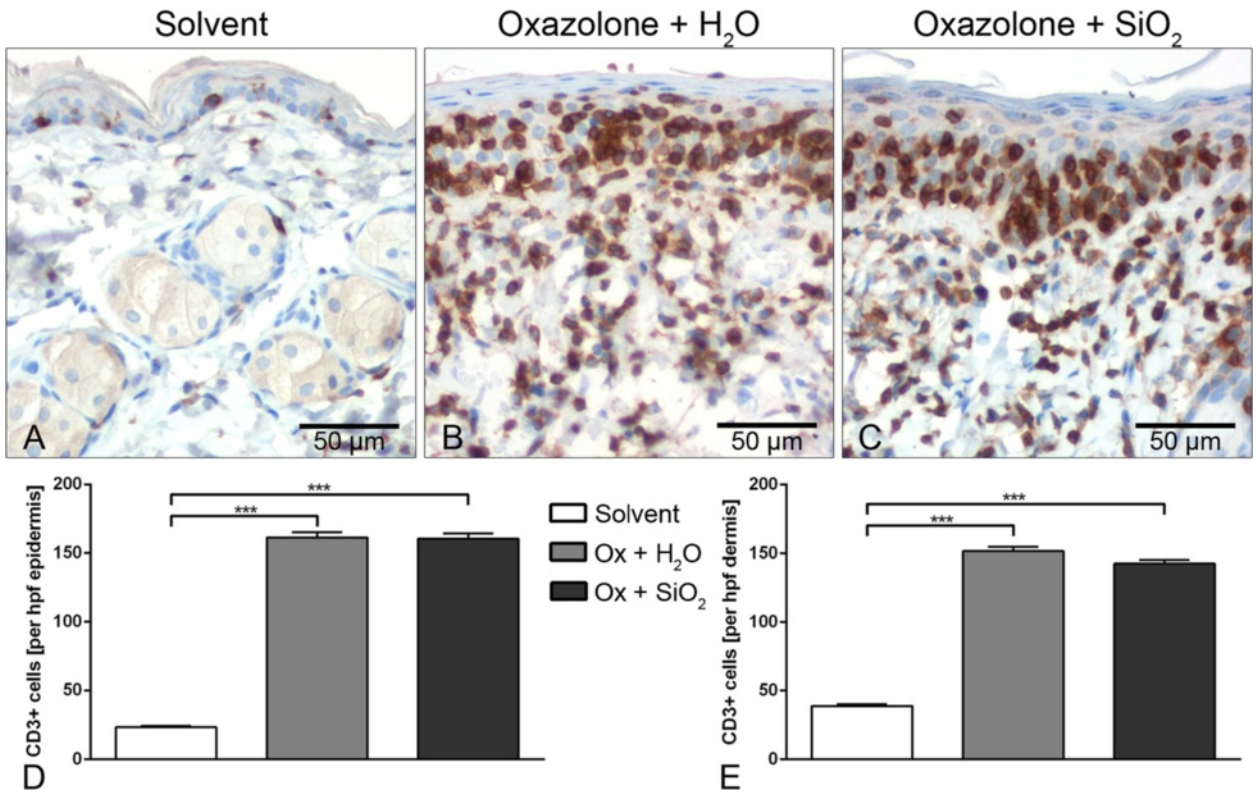

Figure 5 Quantification of T lymphocytes. Cellular infiltrates of the skin in acute contact dermatitis were mainly attributed to T lymphocytes as identified immunohistochemically by the detection of CD3 antigen and visualized with the brown chromogen diaminobenzidine. Nuclei were counterstained with hemalaun. Significant differences were observed only between the acetone solvent control **(A)** and oxazolone (Ox)-treated groups but not between the vehicle control **(B)** and AHAPS-SiO_2-NP-treated groups **(C)**. The same quantitative effects were seen for T lymphocytic infiltration into the epidermis **(D)** and dermis **(E)**. Data are presented as mean ± SEM; Ox-treated groups: $n = 5$; solvent-treated group: $n = 3$; ***$p < 0.001$.

impermeable stratum corneum [23] especially when the thickness is increased due to hyperkeratosis. External, topical application of NP was reported only in a single study so far [7] in which a mild aggravation of skin lesions was observed. However, in that study, the SiO_2-NP were applied simultaneously with mite antigen over a 4-week period with three applications per week. So far, the effects of topical SiO_2-NP exposure to an already existing ACD have not been studied although ACD is a common skin disorder in Western European and North American people with a prevalence of about 20% [9]. As reported by several studies, even higher values for IgE, mast cells, eosinophils, and CD3-positive cells could have been expected in inflamed mouse skin. It therefore seems likely that NP-induced aggravation of the ACD would still have been detectable in the background of our Ox-induced inflammation [5,24-26]. Furthermore, it has been shown that surface functionalization improves the biocompatibility of SiO_2-NP [10,11]. Therefore, both the failure of penetrating beyond the stratum corneum and the improved biocompatibility due to functionalization may have prevented the aggravation of barrier defects and inflammatory response in our study. However, we cannot exclude the possibility that effects would have occurred if significantly

Table 1 Morphometry and quantification of data from lesional skin

	Solvent	Oxazolone + H_2O	Oxazolone + AHAPS-SiO_2-NP
Epidermal thickness [µm]	23.04 ± 1.21	68.95 ± 1.7	61.84 ± 2.58
Mast cells[a]	14.08 ± 0.51	25.34 ± 0.9	23.84 ± 0.86
Eosinophils[a]	0.93 ± 0.12	8.30 ± 0.24	7.63 ± 0.23
T lymphocytes[a] in the epidermis	23.33 ± 1.05	161.36 ± 3.87	160.52 ± 3.65
T lymphocytes[a] in the dermis	38.63 ± 1.33	151.52 ± 3.19	142.54 ± 2.71

[a]Number of cells per high-power field (hpf), counted in at least 10 hpf. Epidermal thickness measurements and quantification of mast cells, eosinophils, and CD3+ T lymphocytes in the epidermis and dermis of lesional skin expressed as mean ± SEM.

higher doses would have been used. Still, the dose used here appears to reflect a realistic condition and offers optimal comparability with the previous studies on unfunctionalized and functionalized SiO_2-NP [5,8,17].

In addition, the specific model used here for the induction of ACD may have had an effect on the results. Ox-induced dermatitis cannot be strictly classified as either T helper (Th)1- or Th2-dominated response [27]. In contrast, ovalbumin and mite antigens result in specifically Th2-driven allergic dermatitis. However, the specific roles of the immune mechanisms involved, both in terms of induction of the hypersensitivity reaction and the exacerbation of allergic disease by certain NP other than AHAPS-SiO_2-NP, need to be studied in the future.

Conclusions

Taken together, our data show that AHAPS-SiO_2-NP exposure to diseased skin in an ACD model does not affect the course and outcome of the disease over 5 days. It thus seems that a short-time exposure of AHAPS-SiO_2-NP to mouse skin is without any pathological consequences, at least as far as can be judged with the techniques employed here. The reasons why the AHAPS-functionalized NP do not modulate barrier disruption or inflammatory responses as seen in other allergic disease models and whether the observations hold true in a long-term exposure model should be addressed in the future.

Abbreviations

ACD: Allergic contact dermatitis; AHAPS: *N*-(6-aminohexyl)-aminopropyltrimethoxysilane; DAPI: 4',6-diamidino-2-phenylindole; ELISA: Enzyme-linked immunosorbent assay; FITC: Fluorescein-5-isothiocyanate; HE: Hematoxylin and eosin; IgE: Immunoglobulin E; NP: Nanoparticles; Ox: Oxazolone; PEG: Polyethylene glycol; SiO_2-NP: Silica nanoparticles; TEWL: Transepidermal water loss; Th: T helper cells.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AO carried out the animal experiments, participated in the design of the study; conducted the histologic, morphometric, and immunohistochemical analyses; performed the ELISA and statistical analyses of all data; and drafted the manuscript. DN, CG, and ER synthesized, characterized, and provided AHAPS-SiO_2-NP and helped to draft the manuscript. LM helped with the necropsy of animals, participated in the design of the study and data analyses, and helped to draft the manuscript. JWF and JL gave conceptual advice and participated in the design of the study. ADG supervised the project, participated in the design of the study, and helped to draft the manuscript. All authors discussed the results and commented on the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank Alexandra Harder and Michaela Dauer for the excellent technical support. This work was funded by the German Research Foundation (DFG) Priority Program 1313 Biological Responses to Nanoscale Particles Cluster NANO-SELECT and the DFG SFB1112 Projects B02 and C03. This article is part of the PhD thesis of AO.

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Received: 3 July 2014 Accepted: 16 September 2014
Published: 24 September 2014

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doi:10.1186/1556-276X-9-524
Cite this article as: Ostrowski *et al.*: AHAPS-functionalized silica nanoparticles do not modulate allergic contact dermatitis in mice. *Nanoscale Research Letters* 2014 **9**:524.

3 Concluding Discussion

NP have a defined size less than 100 nm in at least one dimension (Crosera et al. 2009). In comparison to their bulk material, NP possess a higher biological activity due to their higher surface area per mass unit (Oberdörster et al. 2005). As a result of their unique characteristics, the fields of application for NP are numerous. Among inorganic NP, SiO_2-NP are a promising candidate in the development of new NP tools in biomedicine due to versatile modification opportunities and their superior biocompatibility (Helle et al. 2013; Slowing et al. 2008; Wang et al. 2008). Possible applications of SiO_2-NP include their use in cosmetics, in cancer therapy and as drug delivery system (Napierska et al. 2010). Following surface modification with the aminosilane group AHAPS, DNA can be bound to the NP and SiO_2-NP are thought to become a promising gene delivery system (Graf et al. 2012; Kneuer et al. 2000a; Kneuer et al. 2000b; Kumar et al. 2004; Luo and Saltzman 2005). Consequently, even particle-based transcutaneous vaccination may be conceivable for SiO_2-NP (Papakostas et al. 2011).

The skin is the largest organ of the body and a potential site of entry for topically applied substances. The skin is a very efficient barrier protecting the organism from the surrounding environment (Crosera et al. 2009). The main barrier function is provided by the SC (Breternitz et al. 2007). In diseased skin conditions, the barrier function of the skin is impaired (Prow et al. 2011). One important disease is ACD which affects about 20 % of the people in western countries, i.e., Europe and North America (Thyssen et al. 2007). Consequently, skin barrier disruptions are quite common in humans.

In recent years, the influence of different skin barrier disruptions on the penetration of inorganic NP has been discussed controversially (Labouta and Schneider 2013). For a complete risk assessment of a new NP species intended for its use in medical applications, the penetration behavior and its performance in intact as well as in diseased skin conditions has to be evaluated (Baroli 2010). Especially under diseased conditions, when the skin barrier is impaired, an increased skin penetration of NP might be the consequence (Prow et al. 2011). Penetrating NP may reach viable epidermal or dermal cells and may be taken up by cells of the immune system, such as LC (Crosera et al. 2009). Furthermore, some studies have suggested an adjuvant capacity of SiO_2-NP in immune mediated skin diseases (Hirai et al. 2012b; Hirai et al. 2011; Hirai et al. 2012c).

Thus, the first aim of this study was to characterize the effects of skin barrier disruptions on the local penetration and systemic distribution of AHAPS-functionalized SiO_2-NP. Although

inconsistent results have been published, it can be assumed that a healthy skin offers good protection against possible penetration of inorganic NP larger than 36 nm (Baroli 2010). Moreover, the results on the penetration through an impaired skin barrier have also been discussed contradictorily in the literature on inorganic NP. As reviewed by Labouta and Schneider, the high diversity in different particles used in terms of their material, shape, size, and surface charge make it difficult to compare those studies and to draw general conclusions on the penetration of inorganic NP. Furthermore, the experimental setups (i.e., *in vivo* versus *in vitro,* human study versus animal model) and read-out systems even complicate this comparison (Labouta and Schneider 2013).

As a second aim, the course and outcome of an AHAPS-SiO_2-NP exposure during an existing ACD was characterized. Several studies on inorganic NP, including, SiO_2-NP, TiO_2-NP and polystyrene NP, revealed an aggravation of the inflammatory response in mouse models when NP and allergen were applied concurrently (Hirai et al. 2012b; Hirai et al. 2011; Hirai et al. 2012c; Yanagisawa et al. 2009; Yanagisawa et al. 2010). Despite the high prevalence of ACD, no study has addressed the disease outcome in an already inflamed skin condition following NP exposure.

3.1 Effect of Different Skin Barrier Disruptions on the Local Penetration and Systemic Distribution of Silica Nanoparticles

In recent years, the skin penetration of inorganic NP has been discussed controversially. The discrepancy between studies reporting a particle penetration and those that did not see a penetration might be explained by the multitude of NP species, experimental designs and detection methods employed (Labouta and Schneider 2013). These and other factors, such as vehicle used as well as NP concentration make it very difficult to compare the studies. Furthermore, some authors suspect a higher chance to publish paper demonstrating a dermal penetration of NP. This was explained by a supposed higher attractiveness of these reports to the research community (Labouta and Schneider 2013). However, the attractiveness of studies yielding no particle penetration should not be neglected. Especially in NP application where no penetration is desired, for example, as topical drug delivery system, in cosmetic or sunscreen, these “negative” results might be good arguments to justify the use of NP and reduce public concerns using NP-based products in every-day life.

In accordance with several reports on other inorganic NP, AHAPS-functionalized SiO_2-NP with a size of about 55 nm did not penetrate the intact murine skin in this study. However, the two *in vivo* studies on the topical treatment of mice with unfunctionalized SiO_2-NP revealed a penetration of 70 nm SiO_2-NP after three or 28 days of treatment (Hirai et al. 2012a; Nabeshi

et al. 2011b). At least in one of these studies, 10 % isopropyl myristate was used as vehicle. This chemical compound is nearly insoluble in water (Mistral Industrial Chemicals 2014) and interacts with the lipids of the SC. This interaction results in a modified lipid arrangement (Engelbrecht et al. 2012) and might result in altered diameter of lipid channels in the SC. Under physiological conditions, it can be assumed that the lipid channels of the SC are between 50 and 100 nm whereas the pathways for hydrophilic penetration have a diameter of about 20 nm (Lawson et al. 2007). This probably can explain the divergent findings, although Labouta and colleagues stated for lipophilic vehicles being more a contributing factor than being the main factor in particle penetration (Labouta et al. 2011).

However, following tape stripping that removes the SC with its lipids, no penetration of AHAPS-functionalized SiO_2-NP was observed. Due to missing systematic *in vivo* studies on SiO_2-NP penetration through a disrupted skin, the results of this study have to be discussed with results on other NP species, such as QD, TiO_2-NP or ZnO-NP. The group of Gopee demonstrated a penetration of QD through murine skin only following dermabrasion but not in intact skin or following tape stripping (Gopee et al. 2009). Those results are consistent with the *in vitro* findings of Zhang studying QD penetration in rat skin (Zhang and Monteiro-Riviere 2008) and Senzui studying TiO_2-NP in porcine skin (Senzui et al. 2010). However, Rancan and her colleagues revealed a penetration of 42 nm APS-functionalized SiO_2-NP and a cellular association with keratinocytes and LC after cyanoacrylate stripping of excised human skin (Rancan et al. 2012). This finding was explained by direct contact between NP and viable epidermal cells. However, similar as the AHAPS- SiO_2-NP, the APS-functionalized SiO_2-NP had also a positive zeta potential and were dissolved in an aqueous vehicle. A possible explanation might be a changed barrier integrity of excised human skin as the result of hypoxia as well as the cyanoacrylate stripping which is thought to be more invasive than tape stripping.

Despite their high prevalence, inflammatory skin diseases have been neglected in terms of their influence on NP penetration. The two available *in vivo* studies revealed partially contradictory findings. The first assessed the penetration of 35 nm ZnO-NP through the lesional skin of human patients with AD (Lin et al. 2011). In that no penetration was observed. In contrast to this, Ilves employed a murine AD model and showed a penetration of 20 nm ZnO-NP into the viable epidermis and dermis (Ilves et al. 2014). The AHAPS-functionalized SiO_2-NP in this study did not reach the viable epidermis or even the dermis. These inflicting results on AHAPS-SiO_2-NP and ZnO-NP might be explained by the smaller size of penetrating NP. Besides this, in the study of Ilves a tape stripping of AD skin prior particle application was performed to mimic scratching behavior of AD patients and the skin was further occluded during particle application (Ilves et al. 2014). This additional injury might be a possible explanation for the observed penetration of ZnO-NP, too. Moreover, the occlusion of the skin may also have a

tremendous influence on the skin barrier integrity. It was stated that an occlusion of irritated skin resulted in higher TEWL values compared to non-occluded controls (Jungersted et al. 2010). An ultrastructural analysis demonstrated that occlusion had no effects on intact skin but an occlusion following tape stripping prevented the lipids of the SC being processed into their physiological lipid bilayers (Jiang et al. 1998). Thus, it is likely that the occlusion promoted penetration of ZnO-NP in the study of Ilves and colleagues. Furthermore, the absence of AHAPS-SiO_2-NP penetration in this project, independent of the kind of skin barrier disruption, may also be explained by an increased synthesis of lipids, DNA and proteins as well as the increase in skin pH in both conditions, tape stripping and ACD (Ahn et al. 2001; Man et al. 2007; Wood et al. 1996). In addition to this, in diseased skin the epidermal thickness, metabolic capacity and the microstructure of the SC is modified and should be taken into account (Baroli 2010). Due to the few studies regarding the penetration behavior through an inflamed skin condition and the different experimental approaches, no general conclusion can be drawn so far.

The topical treatment of mice as well as the subcutaneous injection of AHAPS-SiO_2-NP did not induce any obvious toxic effects in this study. For unfunctionalized SiO_2-NP, other authors reported an increase in apoptotic cells *in vivo* although appropriate controls were missing (Nabeshi et al. 2011b). Nonetheless, *in vitro* studies suggested that a surface modification with amine or carboxyl groups results in a reduced cytotoxicity in HaCaT cells (Yoshida et al. 2012).

In conclusion, our results suggest that AHAPS-functionalized SiO_2-NP seem to be a quite safe nanomaterial for both topical application and s.c. injection.

However, using NP for dermal treatment it has to be distinguished between the desired effects: local effects on the skin surface, effects within the skin or a systemic effect. For transdermal drug delivery approaches, the penetration of the NP or its transported drug is usually required (Schroeter et al. 2010). If skin penetration of NP in biomedical approaches is desired, it remains questionable if AHAPS-functionalized SiO_2-NP studied under this conditions are appropriate candidates. Thus, more research has to be done on this field. In order to employ AHAPS-SiO_2-NP as nonviral gene therapy (Luo and Saltzman 2005), an uptake by target cells is required. Those target cells could be LC as the professional APC of the epidermis. Due to high density of LC in hair follicles (Vogt et al. 2006), a follicular targeting of AHAPS-functionalized SiO_2-NP could also be an interesting approach for their future biomedical applications. For positively charged APS-functionalized SiO_2-NP, a follicular accumulation has been reported (Rancan et al. 2012). However, this remains speculative due to missing data on AHAPS-SiO_2-NP.

Although several authors suggested their particles to be used as model particles in penetration studies, it seems that there is not a real model particle allowing for generalized conclusions on

the penetration of NP. Each particle has to be studied on its own with regard of its size, shape and zeta potential, the vehicle used, the experimental setup, detection methods employed and other relevant factors. Those factors and the contradictory results published in literature on same NP species make a systematic comparison between all those studies almost impossible. A further issue is that not all NP used are characterized in the same way. Usually the sizes are given but other important information, such as the zeta potential or the colloidal stability in the vehicle, are missing. It would be highly desirable for future studies that at least the particles are characterized as much as possible.

3.2 Effect of AHAPS-Functionalized Silica Nanoparticle Exposure to an Allergic Contact Dermatitis

The second hypothesis was that AHAPS-functionalized SiO_2-NP aggravate the inflammatory response in the ACD mouse model. Synthetic NP can be designed with the purpose to target or avoid interactions with the immune system. Usually, NP are taken up by macrophages as part of the innate immune response, which is the cornerstone of interactions with the immune system, and inflammatory reactions may be promoted. On the other hand, an absence of immune response is also described. There are indications that NP improve antigenicity of antigens and that they may act as adjuvant (Zolnik et al. 2010). An enhanced allergic response following NP exposure is assumed to be mediated by the direct inflammatory response towards NP (Nygaard et al. 2009). This has been shown, for example, in the case of unfunctionalized SiO_2-NP in mouse models of AD (Hirai et al. 2012b; Hirai et al. 2011; Hirai et al. 2012c). Others reported even a reduction of AD-like skin lesions in histologic and molecular parameters although the antigen-specific antibodies were increased following a treatment with ZnO-NP (Ilves et al. 2014).

Once again, the physicochemical properties of NP are responsible for their compatibility with the immune system. It is assumed that a hydrophilic environment, such as a polyethylene glycol (PEG)-coating of NP, shields the particles from the immune system (Zolnik et al. 2010). The aminosilane group AHAPS is a hydrophilic coating for NP (Kassir 2013) and a further possible explanation why AHAPS-functionalized SiO_2-NP in this study had no aggravating effect in the ACD. But recently, Brandenberger and her team demonstrated also an adjuvant effect of PEG-coated SiO_2-NP using an Ova-induced allergic airway disease model (Brandenberger et al. 2013). Unfortunately, the zeta potential in that study was not measured and, consequently, also the studies using allergic diseases can barely compared.

However, it has to be kept in mind for future applications that NP-specific antibodies may also affect the efficacy and safety of those delivery systems (Zolnik et al. 2010).

All studies evaluating the outcome of NP exposure in models of allergic diseases applied the NP concurrently with the allergen. This was usually justified by increasing numbers of NP in aerosols and the environment that might overcome barriers and are known to be a risk factor in the development of causing or exacerbating allergic diseases (Brandenberger et al. 2013; Hirai et al. 2012b). It is definitely important to know what possible hazard NP may possess but due to the high prevalence of ACD as well as AD, the effects of NP exposure in an already existing inflammation have to be evaluated as it was done in this study. Furthermore, by injecting the NP intradermally the authors of those studies expect the NP as well as the allergen to overcome the SC and reach viable epidermal layers. But this is an issue which has not been resolved finally so far and is in contrast to our penetration results. Furthermore, the definite immune response mechanisms are still elusive and more data are required. Especially, the role of a Th1- or Th2-dominated immune response, respectively, is not defined so far. There are some suggestions that particles bigger than 1 µm induce a Th1 response and smaller sized particles (< 500 µm) induce a Th2 response (van Zijverden and Granum 2000), but more research is needed to understand the complex network of all contributing factors. The knowledge on the Th immune response towards engineered NP would allow for the development of safer NP for biomedical purposes (Dobrovolskaia and McNeil 2007).

3.3 Conclusions

In the first part of this study, the influence of skin barrier disruptions on the local penetration and systemic distribution of AHAPS-functionalized SiO_2-NP were studied following a single application or treatment period of five days. It could be shown that well dispersed and colloidal stable AHAPS-SiO_2-NP were unable to penetrate the skin beyond the SC in intact skin. Even if the skin barrier is disrupted mechanically or by inflammation, the particle did neither overcome the outer layer of the SC or reach viable epidermal cells which is in contrast to the data published on unfunctionalized SiO_2-NP (Hirai et al. 2012a; Nabeshi et al. 2011b).

In addition to this, injected AHAPS-functionalized SiO_2-NP were mainly taken up by macrophages that are scavenger cells as well as APC. Only after the treatment period of five days, AHAPS-SiO_2-NP were localized in the RLN. The immunofluorescent detection of the surface marker major histocompatibility complex (MHC) class II as well as the ultrastructural analysis of the RLN suggested a transport of the SiO_2-NP via APC to the RLN.

However, despite the intracellular location of AHAPS-SiO_2-NP following s.c. injection no toxic effects except the local infiltration with immune cells at the site of injection were observed.

Moreover, as revealed in the second part of this study, AHAPS-functionalized SiO_2-NP seem not to modulate the course and outcome of an ACD. This is contrastive as it has been reported

for unfunctionalized SiO_2-NP in models of AD where NP and the allergen are co-administered (Hirai et al. 2012b; Hirai et al. 2011; Hirai et al. 2012c). In the study on AHAPS-SiO_2-NP, the NP were applied independent of the hapten oxazolone. The histological parameter as well as the IgE content in the serum of the mice revealed no significant differences between the particle-treated group and the control animals.

In summary, it can be stated that the skin barrier provides outstanding protection against the penetration of AHAPS-functionalized SiO_2-NP. Even in conditions in which the skin barrier is perturbed, no particle reached viable epidermal layers. Furthermore, the exposure to these SiO_2-NP either after topical application or injection seems not to induce serious toxic side effects. The data further suggest that the ACD in the mouse model is not modulated by the particle treatment. However, for biomedical applications a particle penetration is usually desired. Thus, further studies are required to use the AHAPS-functionalized SiO_2-NP as a tool in biomedicine.

3.4 Outlook

Starting with the particles, further studies should investigate the role of the surface modification. The systematic comparison between unfunctionalized and AHAPS-functionalized SiO_2-NP in terms of topical application should be conducted under identical conditions. In order to promote AHAPS-functionalized SiO_2-NP being a biomedical tool, other conditions as employed in this study are necessary. Hence, a different vehicle might not remove the lipids of the SC but a disorganization in the lipid ultrastructure (Engelbrecht et al. 2012) may enhance the particle penetration. SiO_2-NP have always thought to be a promising drug or gene delivery system as well as a potential candidate in transdermal vaccination. But all these applications require a skin and / or a follicular penetration and subsequent cellular uptake of NP. Thus, the use of other vehicles have to be tested either with or without further enhancement methods. In a positive case, the controlled release of drugs should be studied as therapeutic system. Therefore, pharmaceutically active substances, for example, anti-inflammatory agents, have to be bound to the particles. Subsequently, the therapeutic benefit should be tested under diseased conditions, whether a reduction of the substance or even a depot function of those carrier systems can be achieved.

Furthermore, SiO_2-NP are known to absorb into cuticular lipids of ectoparasites. These physical interaction leads to a desiccation of the parasites (Barik et al. 2008). Thus, it can be imagined that SiO_2-NP may be used to treat parasitic infections without the risk of resistance development. In this case, no system uptake of NP would be desired. This application would be wanted especially in the development of veterinary products without having concerns on

chemical residues in food producing animals and their products. Moreover, it should be investigated if a SiO_2-NP-treatment also works for endoparasites where resistances to anti-parasitics play an even higher role. Despite their potential use in veterinary medicine, human systemic parasitic infections of high relevance, such as malaria, may also be of great interest in public health issues (Barik et al. 2008).

Another aspect of high interest in future studies is to explore the capacity of AHAPS-SiO_2-NP in vaccine development. As explained, several NP have shown high adjuvant properties resulting in the production of more antigen-specific antibodies or reduced amounts of required antigen for the induction of appropriate immune response compared with the classical adjuvants (Dobrovolskaia and McNeil 2007). However, the correlation of *in vitro* data with *in vivo* data concerning the immune response including their immunotoxicity is limited (Dobrovolskaia and McNeil 2013). Some authors suggested the implementation of *in vivo* studies at an early development stage (Luo and Saltzman 2005) although even with those data an extrapolation to humans is challenging, justified by species-specific differences in the immune system. Thus, the appropriate tests and testing species have to be chosen carefully depending on the future purpose of NP (Dobrovolskaia and McNeil 2013).

However, such further developments require reliable toxicity data on the promising SiO_2-NP candidates. Therefore, the systemic toxicity has to be tested in detail and even more the results concerning a long-term treatment in topical as well as in systemic application require more data. So far, no data on the systemic toxicity of AHAPS-SiO_2-NP are available. These particles should be first evaluated in terms of their potential to induce cytotoxic or genotoxic effects *in vitro* and in the second step also *in vivo*. For all those studies, the detection methods have to be well chosen with respect to their sensitivity. Although no penetration beyond the SC following topical treatment or systemic distribution beyond the RLN following subcutaneous injection were shown by imaging methods, elemental analysis on whole homogenized tissues would be more sensitive to detect small amount of NP in tissues. As an additional aspect, the pharmacokinetic of SiO_2-NP within the organism with all possible excretion pathways should be characterized for NP reaching the systemic circulation.

It can be summarized that several aspects on SiO_2-NP with or without a surface functionalization can be explored to open new opportunities using those NP in biomedicine.

4 Summary

Silica Nanoparticles in Mouse Models of Skin Diseases: Local Penetration, Systemic Distribution and Effect on Allergic Contact Dermatitis

Anja Ostrowski

Nanoparticles (NP) with a defined size less than 100 nm in at least one dimension possess completely new characteristics in comparison to the corresponding bulk material. The new characteristics are currently thought to enable various new applications. Thus, NP can nowadays be found in food, cosmetics and clothes as well as in electronic devices and paints. Recent developments led to biomedical applications, including drug delivery systems, anti-tumor therapy and medical imaging tools. Among promising candidates for further progress are silica nanoparticles (SiO_2-NP). Among the various modification opportunities, a surface functionalization with *N*-(6-aminohexyl)-aminopropyltrimethoxysilane (AHAPS) has been successfully employed for subsequent DNA binding and gene delivery *in vivo*. However, the new properties of NP are not only promising for novel applications, they also have to be considered having a distinct biological behavior with potential unwanted or even toxic effects.

The skin as the largest organ of the body is an important interface between the organism and intended as well as unintended NP contact. The penetration of NP through intact skin has been discussed controversially in recent years. In addition, skin barrier disruptions are very common. For example, allergic contact dermatitis (ACD) affects about 20 % of the people in western countries. But similar as for intact skin, studies on the penetration behavior of NP through a disrupted skin barrier have revealed contradictory results, too. However, most studies employed physical methods to induce a disruptive skin barrier. Only very few authors investigated the penetration of NP in inflammatory skin conditions. Moreover, several studies demonstrated an adjuvant capacity of NP, including unfunctionalized SiO_2-NP. It was shown that SiO_2-NP exposure during allergen sensitization resulted in an aggravation of inflammatory reaction in models of allergic skin diseases. However, no data on the outcome of an ACD exist when allergen and NP exposure are independent from each other.

Consequently, in this *in vivo* study the role of skin barrier disruptions on the penetration of fluorescently labeled AHAPS-functionalized SiO_2-NP was studied comparing a mechanical skin barrier disruption and an inflammatory skin disease with intact skin. As model for a mechanical skin barrier injury, the stratum corneum was removed by tape stripping. In case of the inflammatory skin disease, an ACD was induced with oxazolone. Following successful barrier perturbation, animals were treated with AHAPS-functionalized SiO_2-NP with a single

application or five treatments on five consecutive days, respectively. Controls received the AHAPS-SiO_2-NP on intact skin and in two further groups the particles were injected subcutaneously. In all topically treated groups, AHAPS-functionalized SiO_2-NP were only localized extracellular between the sheet of the stratum corneum despite severe barrier disruptions using fluorescence and transmission electron microscopy. No particles were detected in viable epidermal layers or in the dermis. Following subcutaneous injection, light microscopic examination revealed a moderate infiltration of immune cells, mainly macrophages, at the site of injection. All SiO_2-NP showed a cellular association. The particles were mainly taken up by macrophages which were identified using specific markers. Only following five subcutaneous injections on five consecutive days, AHAPS-functionalized SiO_2-NP were localized in the regional lymph node. The immuno-fluorescent as well as electron microscopic investigation suggested a particle transport to the lymph nodes via antigen-presenting cells. In addition to these findings, the light and electron microscopic analyses of the mice revealed no toxic effects in the macrophages of the draining lymph node despite an intracytoplasmic particle localization.

In addition to the particle penetration behavior, the course and outcome of an ACD mouse model was studied in the presence of AHAPS-SiO_2-NP. In addition to the clinical and histopathologic evaluation, the morphometric data, i.e., epidermal thickness, number of mast cells, eosinophils and CD4 positive cells, and the immunoglobulin E content in the serum revealed no significant differences between both treatment groups of ACD.

Taken together, our results suggest that AHAPS-functionalized SiO_2-NP are unable to penetrate intact and barrier disrupted skin beyond the stratum corneum. Moreover, no modulation of inflammatory reaction was observed in a mouse model of ACD. Consequently, other conditions or application routes have to be investigated in order to employ AHAPS-SiO_2-NP in biomedical applications where a transdermal penetration is intended. As shown for subcutaneous injection, a five-day-treatment period resulted in a transport of AHAPS-functionalized NP to the regional lymph node presumably via antigen-presenting cells. It seems that the AHAPS-SiO_2-NP are inert particles which are treated by the body as insoluble foreign bodies. However, if no penetration of particles is desired, the conditions studied here might be a good start for further investigations.

5 Zusammenfassung

Silica Nanopartikel in Mausmodellen von Hauterkrankungen: Lokale Penetration, Systemische Verteilung und Einfluss auf allergische Kontaktdermatitis

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Nanopartikel (NP) mit einer definierten Größe unter 100 nm in mindestens einer Dimension weisen völlig neue Eigenschaften im Vergleich zum entsprechenden, größer skalierten Grundmaterial auf. Diese neuartigen Eigenschaften ermöglichen den Einsatz in verschiedenen, neuen Anwendungen. So sind NP heutzutage Bestandteil von Nahrungsmitteln, Kosmetika und Kleidungsstücken, finden sich aber auch in elektronischen Geräten und Anstrichfarben. Jüngste Entwicklungen haben zu biomedizinischen Anwendungen, zum Beispiel als Arzneimitteltransporter, Tumortherapeutikum und Einsatz in der bildgebenden Diagnostik, geführt. Ein vielversprechender Kandidat für weiteren Fortschritt sind Silica-Nanopartikel (SiO_2-NP). Unter den verschiedenen Modifizierungsmöglichkeiten hat sich eine Oberflächenfunktionalisierung mit *N*-(6-aminohexyl)aminopropyltrimethoxysilane (AHAPS) für die Bindung von DNA und anschließendem *in vivo* Gentransport als vielversprechend erwiesen. Allerdings sind die neuen Eigenschaften der NP nicht nur vielversprechend für neue Anwendungen, auch ein unterschiedliches biologisches Verhalten mit potenziell unerwünschten oder sogar toxischen Folgen muss in Betracht gezogen werden.

Die Haut ist als größtes Organ des Körpers eine wichtige Grenzfläche zwischen dem Organismus und sowohl beabsichtigtem als auch unbeabsichtigtem NP-Kontakt. Die Penetration von Nanopartikeln durch die intakte Hautbarriere wurde in den letzten Jahren kontrovers diskutiert. Zudem sind Hautbarrierestörungen weit verbreitet. Beispielsweise leiden etwa 20 % der Menschen in westlichen Ländern an einer allergischen Kontaktdermatitis (ACD). Ähnlich wie für die gesunde Haut ergaben Studien zum Penetrationsverhalten von NP durch eine gestörte Hautbarriere widersprüchliche Ergebnisse. Hinzu kommt, dass in den meisten dieser Studien physikalische Methoden zur Störung der Hautbarriere angewendet wurden. Nur wenige Autoren untersuchten das Penetrationsverhalten von NP in entzündlichen Hauterkrankungen. Ferner zeigten mehrere Studien eine Adjuvans-Wirkung von NP, unter anderem auch für unfunktionalisierte SiO_2-NP. So konnte nachgewiesen werden, dass eine SiO_2-NP-Exposition während der Allergensensibilisierung zu einer verstärkten Entzündungsreaktion allergischer Hauterkrankungen im Mausmodell führte. Jedoch fehlen Daten zum

Ausgang einer ACD, wenn die Allergensensibilisierung und die SiO_2-NP-Exposition unabhängig voneinander erfolgen.

Folglich wurde in dieser *in vivo* Studie die Rolle von Hautbarrierestörungen auf die Penetration von fluoreszenzmarkierten AHAPS-funktionalisierten SiO_2-NP untersucht, indem eine mechanische Hautbarrierestörung und eine entzündliche Hauterkrankung mit dem Penetrationsverhalten in intakter Haut verglichen wurden. Als Modell einer mechanischen Hautbarrierestörung wurde das *Stratum corneum* mittels Klebestreifen entfernt. Im Fall der entzündlichen Hauterkrankung wurde eine ACD mit Oxazolon induziert. Nach erfolgreicher Barrierestörung erhielten die Tiere die AHAPS-funktionalisierten SiO_2-NP einmalig beziehungsweise fünf Behandlungen an fünf aufeinander folgenden Tagen. Die Kontrollen erhielten die AHAPS-SiO_2-NP auf die intakte Haut und zwei weiteren Gruppen wurden die Partikel subkutan injiziert. In allen topisch behandelten Gruppen konnten trotz schwerwiegender Barrierestörung die AHAPS-funktionalisierten SiO_2-NP nur extrazellulär zwischen den Lagen des *Stratum corneums* mittels Fluoreszenz- und Transmissionselektronenmikroskopie lokalisiert werden. Keine Partikel konnten in den lebenden epidermalen Schichten oder der Dermis detektiert werden. Nach subkutaner Injektion ergab die histologische Untersuchung der Injektionsstelle eine moderate Infiltration mit Immunzellen, vorrangig Makrophagen. Alle SiO_2-NP zeigten eine zelluläre Assoziation. Die Partikel wurden überwiegend von Makrophagen aufgenommen, welche mittels spezifischen Markern identifiziert wurden. Erst nach fünf Injektionen an fünf aufeinander folgenden Tagen konnten die AHAPS-funktionalisierten SiO_2-NP im regionalen Lymphknoten lokalisiert werden. Die Immunfluoreszenz und die elektronenmikroskopische Untersuchung deuten auf einen Partikeltransport in die Lymphknoten via antigenpräsentierende Zellen hin. Zudem ergaben sich aus der licht- und elektronenmikroskopischen Untersuchung der Mäuse keine Hinweise auf toxische Effekte in den Makrophagen der drainierenden Lymphknoten keine Hinweise auf toxische Effekte trotz intrazytoplasmatischer Partikellokalisation.

Neben dem Penetrationsverhalten der Partikel wurde der Verlauf und Ausgang einer ACD im Mausmodell untersucht. Zusätzlich zu der klinischen und histopathologischen Untersuchung konnten keine signifikanten Unterschiede zwischen beiden Behandlungsgruppen der ACD in den morphometrischen Daten, das heißt epidermale Dicke, Anzahl der Mastzellen, eosinophilen Granulozyten und CD4 positiven Zellen, sowie dem Gehalt an Immunglobulin E im Serum festgestellt werden.

Zusammenfassend sprechen die Ergebnisse dieser Studie dafür, dass AHAPS-funktionalisierte SiO_2-NP nicht in der Lage sind, eine intakte oder gestörte Hautbarriere über das *Stratum corneum* hinaus zu überwinden. Zudem konnte keine Beeinflussung auf die Entzündungsreaktion im Mausmodell einer ACD beobachtet werden. Als Konsequenz lässt

sich daraus schließen, dass für eine Verwendung von AHAPS-SiO_2-NP in biomedizinischen Anwendungen, in denen eine transdermale Penetration angestrebt ist, andere Bedingungen oder Applikationswege in Betracht gezogen und untersucht werden müssen. Beispielsweise transportierten mutmaßlich antigenpräsentierende Zellen nach fünftägiger subkutaner Injektion die AHAPS-funktionalisierte SiO_2-NP in den regionalen Lymphknoten. Es scheint, dass AHAPS-SiO_2-NP inerte Partikel sind, die vom Körper wie nicht lösliche Fremdkörper behandelt werden. Wird jedoch eine Anwendung angestrebt, in der eine Partikelpenetration unerwünscht ist, sind die hier untersuchten Bedingungen ein guter Ansatzpunkt für weitere Untersuchungen.

6 References

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7 List of Own Publications

Research papers in scientifc journals

Ostrowski A, Nordmeyer D, Boreham A, Brodwolf R, Mundhenk L, Fluhr JW, Lademann J, Graf C, Rühl E, Alexiev U, Gruber AD: Skin barrier disruptions in tape stripped and allergic dermatitis models have no effect on dermal penetration and systemic distribution of AHAPS-functionalized silica nanoparticles. *Nanomedicine: Nanotechnology, Biology, and Medicine 2014, 10:1571-1581*, DOI: 10.1016/j.nano.2014.04.004

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Ostrowski A, Nordmeyer D, Mundhenk L, Fluhr JW, Graf C, Rühl E, Gruber AD: AHAPS-funktionalisierte Silica-Nanopartikel penetrieren nicht die Haut und beeinflussen eine allergische Dermatitis im Mausmodell nicht**.** 57th Annual Conference of the German Veterinary Medical Society, Section Veterinary Pathology, Fulda, Germany (08.03.-09.03.2014), Abstract in: *Tierärztliche Praxis* / *Ausgabe K, Kleintiere, Heimtiere; 42(2) - p. A19;* ISSN: 1434-1239

Ostrowski A, Nordmeyer D, Mundhenk L, Fluhr JW, Graf C, Rühl E, Gruber AD: Effects of skin barrier disruptions on the penetration of AHAPS-functionalized silica nanoparticles in the mouse*.* 31st Annual Meeting of the European Society of Veterinary Pathology and European College of Veterinary Pathologists, London, United Kingdom, 04.-07.09.2013, Abstract in: *Journal of Comparative Pathology; 150:1, 2014, p. 90;* ISSN: 0021-9975

Ostrowski A, Nordmeyer D, Mundhenk L, Fluhr JW, Graf C, Rühl E, Gruber AD: Biodistribution of AHAPS-functionalized silica nanoparticles following subcutaneous injection in the mouse. 8th Ph.D. Symposium of the Dahlem Research School, Freie Universität Berlin, Berlin, Germany, 15.07.2013, Abstract in ISBN: 978 3 86387 326 4

Ostrowski A, Nordmeyer D, Mundhenk L, Fluhr JW, Graf C, Rühl E, Gruber AD: Biodistribution of AHAPS-functionalized silica nanoparticles with FITC cores following subcutaneous injection in the mouse. Reporting Colloquium of the German Research Foundation (DFG) Funding Program SPP1313, Fulda, Germany, 11.-13.02.2013

Poster presentations:

Ostrowski A, Nordmeyer D, Mundhenk L, Fluhr JW, Graf C, Rühl E, Gruber AD: Allergic contact dermatitis in mice is not affected or penetrated by AHAPS-functionalized silica nanoparticles. 2nd Joint European Congress of the ESVP, ESTP and ECVP (32nd Annual Meeting of the European Society of Veterinary Pathology and European College of Veterinary Pathologists), Berlin, Germany, 27.-30.08.2014

Ostrowski A, Nordmeyer D, Mundhenk L, Fluhr JW, Graf C, Rühl E, Gruber AD: Welchen Einfluss haben Hautbarrierestörungen auf die Aufnahme und Verteilung von Silica-Nanopartikeln im Mausmodell? 7th Ph.D. Symposium of the Department of Veterinary Medicine and the Dahlem Research School, Freie Universität Berlin, Berlin, Germany, 13.07.2012, Abstract in ISBN: 978 3 86387 153 6

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8 Funding

This work was funded by the German Research Foundation (DFG) Priority Program 1313 “Biological Responses to Nanoscale Particles”, Cluster NANO-SELECT, the Leibniz Graduate School of Molecular Biophysics, the Helmholtz Virtual Institute on “Multifunctional Biomaterials for Medicine” and the DFG Collaborative Research Center (Sonderforschungsbereich) 1112 projects:

B02 to Eckart Rühl, Institute of Chemistry and Biochemistry–Physical and Theoretical Chemistry, Freie Universität Berlin, Germany

B03 to Ulrike Alexiev, Department of Physics, Institute of Experimental Physics, Freie Universität Berlin, Germany and Helmholtz Virtual Institute–Multifunctional Biomaterials for Medicine, Helmholtz-Zentrum Geesthacht, Teltow, Germany

C03 to Achim D. Gruber and Lars Mundhenk; Department of Veterinary Pathology, Freie Universität Berlin, Germany

9 Acknowledgements

First of all, my greatest appreciation goes to Prof. Dr. Achim D. Gruber, Ph.D., chair of the Department of Veterinary Pathology of the Freie Universität Berlin, for providing the research field and the warm welcome in his institute, especially his support and faith in me and the project as well as his encouraging words during my time working at this project.

I would like to thank Dr. Lars Mundhenk for his helpful support with the experiments and data analysis. I am especially grateful for his food for thoughts to form a good idea into a valuable research contribution.

I appreciate the open ears and all helpful advices of Dr. Olivia Kershaw, Sylke Giese und Prof. Dr. Robert Klopfleisch in nearly every situation. A special thanks goes to Lydia Marie König for reminding me that there is a life outside the institute. I would like to thank Aleksandra Zuraw for her patience and all the fun we had and, of course, for her endeavors keeping the right balance in our office. I appreciate the moral support and encouragement of Kristina Dietert and all the sweets she provided. Moreover, I am grateful for all other colleagues of the Institute of Veterinary Pathology accompanying my recent years in the institute. I appreciate their support, the joint lunches and especially the good collaboration.

Tremendous gratitude goes to PD Dr. Joachim Fluhr, who facilitated my San Francisco experience. He and Prof. Dr. Dr. Jürgen Lademann are much appreciated for their support as my supervisors in subject-specific as well as in human issues during my project. Furthermore, I thankfully acknowledge all collaboration partners for their contributions.

Sincerest thanks go to all my friends. I appreciate that they always care for me and my work including all ups and downs and distract me in times I need it. I especially would like to thank André Kleinpeter to make me choosing this way.

Finally, I express my deepest gratitude to my family, especially to my father, for their never ending support and faith in me.

Declaration of Originality

Hereby, I declare that the present thesis has been prepared by myself. I assure that I exclusively used the mentioned sources and facilities.

Berlin, 2014 Anja Ostrowski