



Skin barrier disruptions in tape stripped and allergic dermatitis models have no effect on dermal penetration and systemic distribution of AHAPS-functionalized silica nanoparticles

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Abstract

The skin is a potential site of entry for nanoparticles (NP) but the role of disease-associated barrier disturbances on the path and extent of skin penetration of NP remains to be characterized. Silica nanoparticles (SiO₂-NP) possess promising potential for various medical applications. Here, effects of different skin barrier disruptions on the penetration of *N*-(6-aminohexyl)-aminopropyltrimethoxysilane (AHAPS) functionalized SiO₂-NP were studied. AHAPS-SiO₂-NP (55 ± 6 nm diameter) were topically applied on intact, tape stripped or on inflamed skin of SKH1 mice with induced allergic contact dermatitis for one or five consecutive days, respectively. Penetration of AHAPS-SiO₂-NP through the skin was not observed regardless of the kind of barrier disruption. However, only after subcutaneous injection, AHAPS-SiO₂-NP were incorporated by macrophages and transported to the regional lymph node only. Adverse effects on cells or tissues were not observed. In conclusion, AHAPS-SiO₂-NP seem to not cross the normal or perturbed mouse skin.

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Key words: Tape stripping; Allergic contact dermatitis; Murine model; FLIM; Fluorescence microscopy

Background

Abbreviations: NP, nanoparticles; SiO₂-NP, silica nanoparticles; AHAPS, *N*-(6-aminohexyl)-aminopropyltrimethoxysilane; QD, quantum dots; FITC, fluorescein-5-isothiocyanate; DLS, dynamic light scattering; SC, stratum corneum; TEM, transmission electron microscopy; TEWL, transepidermal water loss; s.c, subcutaneous; RLN, regional lymph node; HE, hematoxylin and eosin; DAPI, 4',6-diamidino-2-phenylindole; PBS, phosphate-buffered saline; FLIM, fluorescence lifetime microscopy; APC, antigen-presenting cell.

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Silica nanoparticles (SiO₂-NP) are among the most promising inorganic nanoparticles (NP) for biomedical applications. In addition to the selective targeting of cancer cells or drug and gene delivery systems, SiO₂-NP are used for ultrasensitive bioimaging and lymph node tracking or may be used as coating for other NP to reduce their toxicity.^{1–4} Furthermore, particle-based transcutaneous vaccination is viewed as a promising application for SiO₂-NP.⁵ SiO₂-NP possess a favorable biocompatibility, are thus considered a relatively benign material¹ and allow for a wide variety of applications following specific chemical and physical modifications. For example, a *N*-(6-aminohexyl)-aminopropyltrimethoxysilane (AHAPS) surface functionalization of SiO₂-NP may allow for subsequent DNA binding.⁶ Such positively charged AHAPS-SiO₂-NP are highly stable in

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biological fluids and mediate enhanced cellular uptake in vitro.⁷ Consequently, a gene transfection model has already successfully been established in vitro and in vivo.^{8,9}

The skin as largest organ with up to 10% of the body mass is a highly effective interface between the organism and external matter including nanoparticles. Several routes of entry through the skin have been proposed for NP. In general, it is hypothesized that NP may reach deeper skin layers either via intercellular or transfollicular routes.^{10,11} However, penetration is thought to be affected by various factors with dermal structure and integrity being of prime importance.¹² In particular, skin diseases with barrier alterations as common conditions in people are considered to be highly relevant for altered NP penetration. For example, allergic contact dermatitis is increasingly diagnosed in people in developed countries with a current prevalence of between 15% and 30%.¹³

To date only unfunctionalized SiO₂-NP (i.e. negatively charged particles with silanolate groups on the surface) have been studied in terms of skin barrier penetration but not in models with a disrupted epidermal barrier. Recent reports have shown penetration of SiO₂-NP with a diameter of 70 nm across intact skin in vivo. These SiO₂-NP have even entered the systemic distribution after treatments for 3 or 28 days.^{14,15} The in vivo use of other inorganic NP tested including quantum dots (QD) has been discussed controversially. Some authors have stated that carboxyl QD may penetrate healthy and damaged skin after exposure to ultraviolet radiation¹⁶ whereas others have only seen poly(ethylene-glycol)-coated QD penetration in seriously damaged skin after dermabrasion.¹⁷ Ex vivo experiments using rat skin have shown similar results with only a disrupted skin barrier allowing for particle penetration.¹⁸ Titanium dioxide NP have penetrated healthy skin into deeper epidermal layers after 60 days of application and even entered systemic distribution.¹⁹ On the other hand, a targeted formulation for NP was used to reduce skin penetration of nanoscale silver in another study²⁰ suggesting that depending on the formulation NP can even be employed to effectively limit skin penetration. An excellent overview of the current understanding of the penetration of different inorganic NP has been compiled by Labouta and Schneider.¹⁰

In the present study, we compared the skin penetration characteristics and systemic distribution of AHAPS-SiO₂-NP in healthy mouse skin versus skin from two different mouse models with altered epidermal barrier function. In these models, the skin was either disrupted by tape stripping²¹ or a hapten-induced allergic contact dermatitis model.²²

Methods

Particle synthesis and characterization

SiO₂-NP cores were synthesized and covalently labeled with fluorescein-5-isothiocyanate (FITC) using a modified Stöber synthesis.²³ A 3-nm dye-free silica shell was grown in a seeded growth process around the cores resulting in particles with a diameter of 55 ± 6 nm (Figure 1).²⁴ The surface of the SiO₂-NP was modified by covalently surface-bound AHAPS resulting in NP of high colloidal stability and positive zeta potential (Table 1).⁷ Functionalization with AHAPS bearing one primary

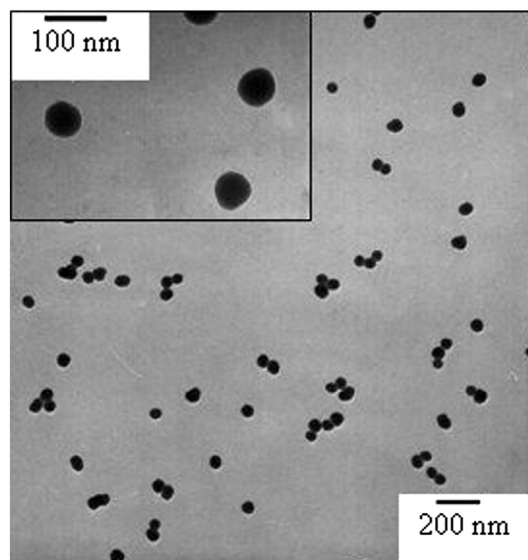


Figure 1. TEM revealed well dispersed FITC-labeled AHAPS-SiO₂-NP with a diameter of 55 ± 6 nm (inset).

and one secondary amino group per molecule changed the zeta potential of the SiO₂-NP from highly negative -45 ± 4 mV to highly positive values (Table 1). For each mouse experiment the SiO₂-NP were freshly functionalized with AHAPS in order to avoid that the amino groups on the particle surface may react with CO₂ that is dissolved in ambient water forming silylated ammonium carbamate salts.²⁵ This process may lead to a reduced zeta potential and, as a consequence, decreased colloidal stability of the NP.

After transfer of the freshly prepared AHAPS-SiO₂-NP from ethanol into ultra-pure water (AlleMan Pharma, Pfullingen, Germany) the NP showed high colloidal stability (Table 1, zeta potential data for all samples were summarized in Table S1; for details of the transfer process see Graf et al.).⁷ In all dynamic light scattering (DLS) measurements the hydrodynamic diameters of the NP remained between 102 nm and 144 nm and the NP zeta potentials remained between $+18$ mV and $+42$ mV (Figure S1). Notably, the particles with a zeta potential of $+18 \pm 1$ mV remained colloidal stable for at least 17 days (sample 5 in Table 2). In parallel to the mouse experiments the AHAPS-SiO₂-NP were measured daily by DLS to control if their colloidal stability changed during the animal experiments (Table 2). In all cases the hydrodynamic diameter remained unchanged.

Animals and study protocol

Six- to eight-week-old, male SKH1 mice (Charles River, Germany) were housed in individually ventilated cages at 20 ± 3 °C and a relative humidity of 35% in a 12-h light/dark cycle with ad libitum access to commercial diet and water. The mice were randomly separated into one of eight groups with $n = 3$ per group. The groups included the treatment on healthy skin for one day (group 1) or treatments over five consecutive days (group 2). A superficial skin barrier disruption was achieved in the right flank area by sequential tape stripping (group 3) removing the stratum corneum (SC) of an area of 1 cm^2 by 129

t1.1 Table 1

t1.2 Transmission electron microscopy (TEM), dynamic light scattering, and zeta potential characterization of AHAPS-functionalized silica nanoparticles.

t1.3	TEM diameter [nm]	Hydrodynamic diameter in ethanol [nm]	Hydrodynamic diameter in ultra-pure water [nm]	Zeta potential in ethanol [mV]	Zeta potential in water for injection [mV]
t1.4	55 ± 6	96 ± 3	111 ± 1	+48 ± 7	+42 ± 2

adhesive tape (Tesa, Hamburg, Germany) until transepidermal water loss (TEWL) values of approximately 40 g/m² h were reached. As second barrier disruption model an allergic contact dermatitis (group 5) was induced with oxazolone (Sigma-Aldrich, Steinheim, Germany) in acetone (Biesterfeld Chemie-distribution, Hamburg, Germany) as reported previously with some modifications.²⁶ Briefly, the mice were sensitized with 1.5% oxazolone in acetone on day 0 on the right flank. One week later a first challenge with 0.5% oxazolone in acetone was applied to the right flank, followed by individual housing of the mice. Twelve hours later (day 7) the AHAPS-SiO₂-NP were applied on the area of inflammation for five consecutive days. On day 9 a second challenge with 0.5% oxazolone in acetone was conducted to ensure a persisting allergic contact dermatitis over the entire study period. Control groups received the solvent (ultra-pure water) either on the tape-stripped skin (group 4) or on the allergic contact dermatitis (group 6). In addition to the topical treatment, we simulated the penetration of the whole nanoparticle dosage by injecting the particles subcutaneously (s.c.) into the right flank once (group 7) or once a day over five consecutive days (group 8).

Each mouse of groups 1 to 6 was treated topically with 250 µg of AHAPS-functionalized SiO₂-NP per day comparable to studies on unfunctionalized SiO₂-NP^{14,15} or the vehicle ultra-pure water, respectively. The treatment was performed on the right flank in isoflurane anesthetized mice (Abbot Laboratories, Maidenhead Berkshire, UK). Following a 5-minute massage with Sanitas® Mini-Massager (Hans Dinslage, Uttenweiler, Germany) the mice were kept under anesthesia for another 55 minutes on a heating mat (Eickemeyer, Tuttlingen, Germany). The solution was allowed to dry and excessive particles were gently wiped off with a paper tissue before animals awoke to prevent oral ingestion by licking. All topically treated mice were housed individually after the beginning of particle application or as stated in experimental design. Prior to each particle application, barrier function was assessed measuring TEWL on the application site with Tewameter TM 300 (Courage + Khazaka, Cologne, Germany) as previously described.²⁷

Twenty-four hours after the last treatment animals were euthanized by cervical dislocation in deep anesthesia and necropsy and sampling of tissues were performed. The study protocol was approved by the State Office of Health and Social Affairs, Berlin (LAGeSo G 0209/11) and conducted according to national guidelines.

t1.4 Histopathologic and immunofluorescence analysis

All tissues for histopathologic analysis including lesional and non-lesional skin and subcutis, regional and non-regional lymph

Table 2

t2.1 Hydrodynamic diameters of AHAPS-functionalized silicon dioxide nanoparticles measured in water for injection before and after the individual mouse experiments.

t2.2	Sample no.	Hydrodynamic diameter before mouse experiment [nm]	Hydrodynamic diameter after mouse experiment [nm]	t2.3
t2.4	1	111 ± 1	111 ± 2	t2.4
t2.5	2	114 ± 1	113 ± 7	t2.5
t2.6	3	106 ± 2	102 ± 1	t2.6
t2.7	4	141 ± 3	143 ± 2	t2.7
t2.8	5	127 ± 4	131 ± 3	t2.8

nodes (RLN), lung, heart, thymus, liver, spleen, kidney, gastrointestinal tract, pancreas, thyroid and adrenal gland, testicles, brain and bone marrow were fixed for 24 h in 4% neutral-buffered formalin. Sections (5 µm) of the formalin-fixed and paraffin-embedded tissues were stained with hematoxylin and eosin (HE) following standard procedures. Serial sections were deparaffinized and counterstained with 4',6-diamidino-2-phenylindole (DAPI; Carl Roth, Karlsruhe, Germany).

To identify the target cells of AHAPS-SiO₂-NP, tissue sections were immunolabeled with antibodies for (a) F4/80 (rat monoclonal [Cl:A3-1] anti mouse F4/80, 1:160, AbD Serotec, Oxfordshire, UK), (b) CD204 (rabbit polyclonal anti-msr1/CD204, 1:50, Bioss, Woburn, USA) or (c) MHC class II (rat monoclonal [M5/114] anti-MHC class II, 1:50, Abcam, Cambridge, UK). Briefly, after deparaffinization endogenous peroxidase was inactivated in 10% hydrogen peroxide in phosphate-buffered saline (PBS). Following antigen retrieval with 0.1% pronase E (AppliChem, Darmstadt, Germany) in PBS and a blocking step with Roti-ImmunoBlock (Carl Roth) in goat serum, sections were incubated with the primary antibodies and subsequently incubated with an Alexa Fluor® 568-labeled secondary antibody (goat anti-rat IgG or goat-anti-rabbit IgG, respectively, 1:200, Life Technologies, Darmstadt, Germany). The slides were counterstained with DAPI as described above. Microscopic examination was conducted with a BX41 microscope (Olympus, Japan) equipped with a digital camera (Color-View II, SIS, Münster, Germany), a fluorescence burner (U-RLF-T, Olympus) and a digital image analysis software (AnalySIS docu, version 5.0, SIS).

Transmission electron microscopy (TEM)

Biopsies of the skin including s.c. tissue of the right and left flanks, of the right inguinal and axillary lymph nodes as well as the left inguinal lymph node were fixed in Karnovsky fixative. The samples were treated according to the general protocols for TEM studies. Embedding and slide preparation was performed as published.²⁸ Ultrathin sections were examined with a Zeiss transmission electron microscope (10CR, Jena, Germany).

Fluorescence life time imaging microscopy (FLIM)

Paraffin sections of healthy skin and s.c. tissue from the right and left flanks of mice (group 8) and of a 2.5 µg/µl AHAPS-SiO₂-NP solution embedded in an agarose gel were mounted on cleaned coverslips (Marienfeld-Superior; Precision cover glasses thickness No. 1.5H) and subjected to FLIM. The time-resolved

Table 3

Transepidermal water loss (TEWL) measurements at the application site prior AHAPS-SiO₂-NP treatment of all animals (mean ± standard error of the mean).

Group	TEWL value (g/m ² h)				
	Day 1	Day 2	Day 3	Day 4	Day 5
Intact skin 24 h*	7.5 ± 0.15	n.d.	n.d.	n.d.	n.d.
Intact skin 5 days*	6.7 ± 0.72	8.8 ± 0.27	7.9 ± 0.32	6.6 ± 1.09	6.7 ± 0.73
Tape stripping [†]	42.9 ± 2.14	n.d.	n.d.	n.d.	n.d.
Allergic dermatitis [†]	13.9 ± 1.36	24.2 ± 5.59	49.7 ± 5.65	63.9 ± 5.24	87.0 ± 2.2

Abbreviations: TEWL, transepidermal water loss; n.d., not determined.

* $n = 3$.

[†] $n = 6$ (treatment group and vehicle control).

fluorescence decay curves in each image pixel were obtained from time-correlated single-photon counting with a confocal laser scanning FLIM setup. The setup consisted of an Olympus IX71 inverted microscope equipped with a 40× objective lens, a confocal scanning unit (DCS-120, Becker & Hickl, Berlin, Germany), and a Ti:Sapphire laser system (Spectra Physics, Santa Clara, USA) in the mode-locked picosecond-pulsed regime.^{29–31} The Ti:Sapphire Tsunami laser was pumped by a solid state Millennia V laser and the laser output was frequency doubled to obtain the excitation wavelength of 486 nm for the fluorescein dye. A pulse picker reduced the repetition rate to 4 MHz. Single-photon counting was performed using a time-correlated single-photon counting (TCSPC) module (SPC-150, Becker & Hickl). The time range was set to 20 ns for 256 channels resulting in a resolution of 78 ps/ch. A colored glass filter (OG515) and a high-quality band-pass filter (HQ525/50) were used as emission filters.

FLIM images were analyzed using self-written routines in C++. Fluorescence decay curves were partitioned into classes (i.e. clusters) using a multivariate pattern recognition method. The fluorescence decays of the individual clusters were fitted with a sum of exponentials using χ^2 minimization. False-color images were generated by assigning a distinct color to all pixels containing a fluorescence decay curve that belonged to one cluster.

Results

Skin barrier disruptions had no effect on particle penetration and systemic distribution

To evaluate the effect of different skin barrier disruptions on the penetration and biodistribution of topically applied AHAPS-SiO₂-NP we compared healthy skin after two different treatment periods (groups 1 and 2) with a mechanically disrupted skin barrier (group 3) and an inflammatory skin barrier perturbation (group 5). The skin barrier was successfully disrupted by sequential tape stripping or the oxazolone induced allergic contact dermatitis as shown by the increased in TEWL values (Table 3 and Figure S2). The skin barriers of animals from group 1 and 2 were intact and within the physiological range as indicated by TEWL values less than 10 g/m² h.²⁷ The topical application in all four particle treated groups resulted in an accumulation of the SiO₂-NP in the superficial layers of the SC on the site of application (Figure 2, A–D) but no SiO₂-NP were

found in the vehicle control groups 4 and 6 or on the contralateral skin of all groups (data not shown). No particles were detected in deeper epidermal or even dermal layers in any of these groups. In all AHAPS-SiO₂-NP-treated groups, particles were detected only very sporadically in hair follicles with no differences between the groups. The TEM analysis supported this finding. In the ultrastructural analysis the AHAPS-SiO₂-NP were traceable extracellularly between the corneal layers of the SC either as single particles or as small clusters (Figure 3, A–F).

Subcutaneously injected AHAPS-SiO₂-NP were taken up by macrophages and transported to the RLN

To simulate complete dermal penetration and to study the systemic biodistribution of AHAPS-functionalized SiO₂-NP the particles were injected subcutaneously into the right flank of the mice once (group 7) or once daily over five consecutive days (group 8). The AHAPS-SiO₂-NP were exclusively detected intracellularly by fluorescence microscopy in cells of the s.c. connective tissue in both treatment groups (Figure 4, A–B). However, no particles were detected in the subcutaneous connective tissue of the contralateral side (Figure 4, C). Immunofluorescent labeling using three different marker antibodies identified macrophages as the major NP uptaking cell type (Figure 4, D–F).

The AHAPS-SiO₂-NP were further localized in RLN only in group 8 (Figure 4, G and I) but not in non-RLN (Figure 4, H). Using MHC class II immunofluorescent labeling antigen-presenting cells (APC) were identified as the target cell of SiO₂-NP within the RLN, suggesting cell-mediated transport of AHAPS-SiO₂-NP to the regional lymph node (Figure 4, J). Fluorescence microscopy failed to detect the AHAPS-SiO₂-NP in any other tissue beyond the RLN. Furthermore, toxicohistopathologic examination of all organs by board certified pathologists (L.M., A.D.G.) yielded no evidence of any pathological changes in the tissues due to the AHAPS-SiO₂-NP except for the immune cell infiltration at the site of injection.

FLIM was used to further characterize the distribution of injected SiO₂-NP in mice from group 8 and to obtain a semi-quantitative estimate of the amount of NP taken up by cells. We compared the different fluorescence lifetime curves from the FLIM images from the left and right flanks of mice of group 8 (Figure 5, A–C). Autofluorescence of tissue (green) was easily distinguished from the fluorescence of erythrocytes (cyan) located in blood vessels (Figure 5, A). However, images from

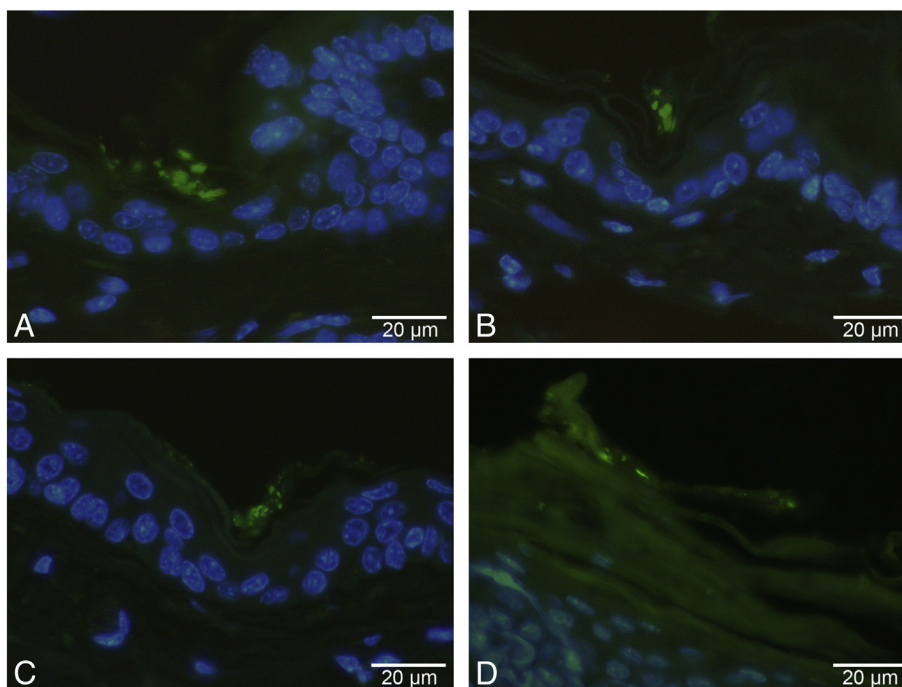


Figure 2. AHAPS-SiO₂-NP failed to penetrate through the stratum corneum (SC) in normal and disrupted skin. FITC-labeled AHAPS-SiO₂-NP were traceable in the SC in the intact skin after a single treatment (A), five treatments over 5 days (B), in the tape-stripped skin (C) and also in the inflamed skin (D). No deeper penetration was observed using fluorescence microscopy. The green signal within nuclei in SiO₂-NP-treated and vehicle control groups (not shown) originated from nucleoli should not be misinterpreted as intranuclear SiO₂-NP.

the tissues of the right flanks (Figure 5, B) contained an additional fluorescence lifetime species (red) that could be distinguished from tissue autofluorescence and was attributed to the fluorescence of AHAPS-SiO₂-NP (Figure 5, C). In agreement with the fluorescence measurements described above, the NP were found only in the s.c. tissue of the sample (Figure 5, B) but were undetectable in the samples from the left flanks, i.e. the contralateral sides. Furthermore, the pixel intensities were much stronger in the regions containing AHAPS-SiO₂-NP (Figure 5, D-E) compared to those without AHAPS-SiO₂-NP indicating intracellular uptake of the NP (Figure 5, D, white rectangle). Increasing the spatial resolution by a factor of 5 allowed for the visualization of individual macrophages containing AHAPS-SiO₂-NP (Figure 5, F). Distinct particle clusters were discernible within these cells. Using a reference sample containing AHAPS-SiO₂-NP at a defined concentration of 2.5 μg/μl the average NP number per pixel was calculated (Figure 5, G). As a result, the clusters within the macrophages contained 15 NP per pixel on average. Since the AHAPS-SiO₂-NP specific clusters inside this macrophage comprised a total of 3626 pixels, the number of NP taken up by a single macrophage was approximated to at least 56,400, corresponding to 9.4 picograms of AHAPS-SiO₂-NP per cell.

Individual or clustered localization of AHAPS-SiO₂-NP in cells of the RLN by TEM

Ultrastructural analysis enabled particle detection on a single particle level and the evaluation of cellular toxic side effects. The AHAPS-SiO₂-NP were localized in cells of the RLN either as

single particles or as groups by TEM analysis but not in the non-RLN (Figure 6, A-C).

The cells containing the particles were in close contact with surrounding lymphocytes via structures consistent with immunologic synapses³² (Figure 6, A), suggesting that the SiO₂-NP uptaking cells were APC. This ultrastructural finding supported the immunofluorescence results identifying the cells to express MHC class II. Despite their intracellular localization we failed to identify any evidence of adverse effects or toxicity related to the particles, e.g., mitochondrial swelling (Figure 6, B).

Discussion

Our data indicate that a superficial skin barrier disruption by tape stripping and a severe skin barrier disruption in an allergic contact dermatitis mouse model may neither lead to an enhanced penetration nor systemic uptake of AHAPS-SiO₂-NP below the SC after topical application. Systematic studies on the penetration of SiO₂-NP through a disrupted epidermal barrier have not been reported to date. Previously, in vivo studies on the penetration of other inorganic NP like QD through intact or disrupted skin barrier have yielded contradictory results. Negatively charged QD of approximately 30 nm in size penetrated intact skin and following a barrier perturbation by ultraviolet radiation more QD reached deeper epidermal layers and even the dermis.¹⁶ Conversely, the penetration and systemic absorption of 37 nm poly(ethylene-glycol)-coated QD were only seen after dermabrasion but not in intact, tape-stripped or acetone-pretreated skin.¹⁷ Of note, lipophilic formulations, such

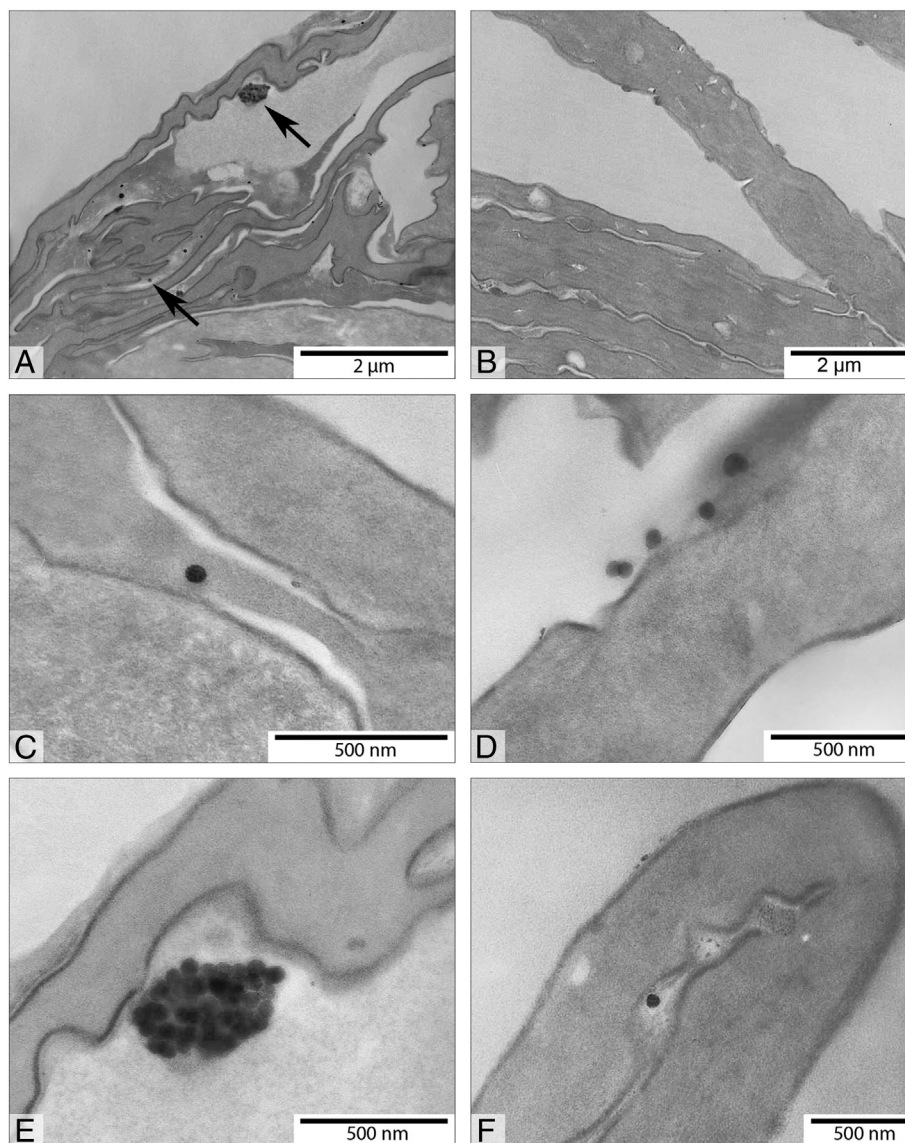


Figure 3. TEM analysis of the skin localized the AHAPS-SiO₂-NP only between the scales of the stratum corneum (SC) regardless of skin barrier disruption. In lower magnifications the particles (arrows) were localized either as clusters or as single particles between the upper corneocytes of the SC (for example, AHAPS-SiO₂-NP-treated mouse after tape stripping, **A**). No NP were detected in vehicle control groups (for example, vehicle control in allergic contact dermatitis, **B**). In all animals with intact skin after 24 h (**C**) and after 5 days (**D**) as well as in tape-stripped skin (**E**) and the allergic contact dermatitis model (**F**) the particles were located extracellularly between corneocytes of the SC. No penetration into deeper epidermal or dermal layers was observed in any of the 16 animals of the 4 groups following external NP application.

as glycerol-containing or an oil-in-water emulsion, were used to enhance penetration of NP in both studies. Others have reported that permeation through the SC via intracellular lipid-channels for lipid-soluble components may be possible up to a size of 50–100 nm whereas the pathway for hydrophilic permeation is limited to particles of approximately 20 nm.³³

To model the mechanical effect during application of topically used drugs in humans and to enhance NP penetration³⁴ we combined massaging of the skin for a short time and two different skin barrier disruption models in our experimental design. In a previous report, an experimental ex vivo setup on human skin resulted in QD reaching deeper skin layers following tape stripping and massaging of the sample to enhance the

penetration of QD.³⁵ The QD in that study had also been dispersed in water but were of a very small size of 3.5 nm. The penetration might have been more an effect of the NP size than of the solvent. Alternatively, opening the hair follicle orifice and damaging the barrier of the hair follicles in excised human skin by cyanoacrylate skin surface stripping has resulted in penetration of negatively charged unfunctionalized and positively charged (3-aminopropyl)-trimethoxysilane-functionalized Stöber SiO₂-NP.³⁶ But even if 110 nm SiO₂-NP in a 5% glucose solution were injected s.c. once no systemic distribution of NP was observed after 24 h or 7 days by imaging techniques.³⁷ Consistent with our result for AHAPS-SiO₂-NP tested in allergic contact dermatitis of mice, an in vivo study focusing on the

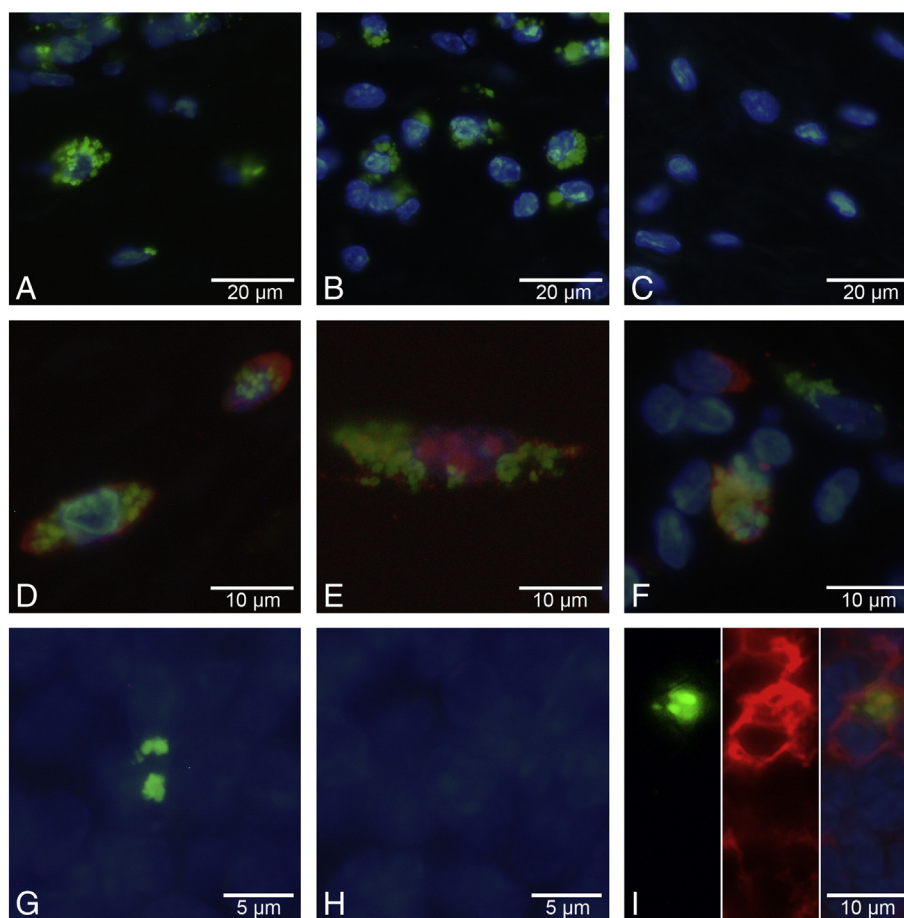


Figure 4. AHAPS-SiO₂-NP were taken up by macrophages after s.c. injection. The AHAPS-SiO₂-NP (green) were localized intracellularly by fluorescence microscopy at the site of injection after 24 h (A) and after five injections over five consecutive days (B) but not in the subcutaneous tissue of the contralateral side (C), blue: DAPI staining of nuclei. The green signal within nuclei in SiO₂-NP-treated and vehicle control groups originates from nucleoli and should not be misinterpreted as intranuclear SiO₂-NP. Immunofluorescent labeling (red) with macrophage markers F4/80 (D), CD204 (E) and MHC class II for APC (F) identified macrophages as major uptaking cells of AHAPS-SiO₂-NP. A spread to the RLN occurred only after five injections over five consecutive days (G), but not after single injection or in non-RLN (H). Immunofluorescent labeling of MHC class II of the RLN suggested a transport to lymph nodes via APC (I), left panel: green fluorescence channel indicating NP, middle panel: red fluorescence channel indicating MHC class II expression and right panel: merge of left and middle panel with blue DAPI counterstain, all three images taken from the same cell.

penetration of 35 nm zinc oxide NP through a disturbed skin barrier localized NP in the SC but NP failed to reach deeper, viable epidermal layers regardless of the kind of skin barrier disruption used (atopic dermatitis or psoriatic skin of human volunteers).³⁸ Increased epidermal thickness and the inflammatory environment of the skin lesions may explain why SiO₂-NP may not penetrate through the inflamed skin despite a severe barrier disruption as confirmed by increased TEWL. However, TEWL is an indirect indicator for the skin barrier integrity and only reflects the “inside–outside” barrier, which not always correlates with the “outside–inside” barrier.³⁹ However, the strong barrier distribution in our models suggests that the outside–inside barrier was also impaired. Tape stripping as well as allergic contact dermatitis may lead to a release and synthesis of lipids, DNA and proteins as well as an increase in skin pH. All these factors may play a crucial role in the permeability of the skin barrier.^{40–43} Clearly, the role and impact of different skin barrier status and skin barrier disruption models in the

penetration of inorganic NP are partially contradictory and needs further classification.¹⁰ As shown previously in a more detailed study on the time course of lymph node arrival of APC, mechanical stress like tape stripping resulted in APC reaching the RLN after approximately 24 h.⁴⁴

AHAPS-SiO₂-NP failed to penetrate healthy skin after 24 h or after a 5-day treatment period in vivo unlike the reported data on unfunctionalized silica nanoparticles with an average hydrodynamic diameter of 77 nm and a negative zeta potential.^{14,15} The impact of different surface functionalizations of SiO₂-NP on dermal penetration in vivo is obviously still poorly understood. The amount of particles used here is comparable to previous studies on unfunctionalized SiO₂-NP but we used a different vehicle. In at least one study analyzing unfunctionalized SiO₂-NP 10% isopropyl myristate, a well-known solubilizer, was used.¹⁵ This could have had an influence on penetration as discussed above. Noteworthy, an aqueous solvent as used in this study allows for the future binding of DNA^{8,9}

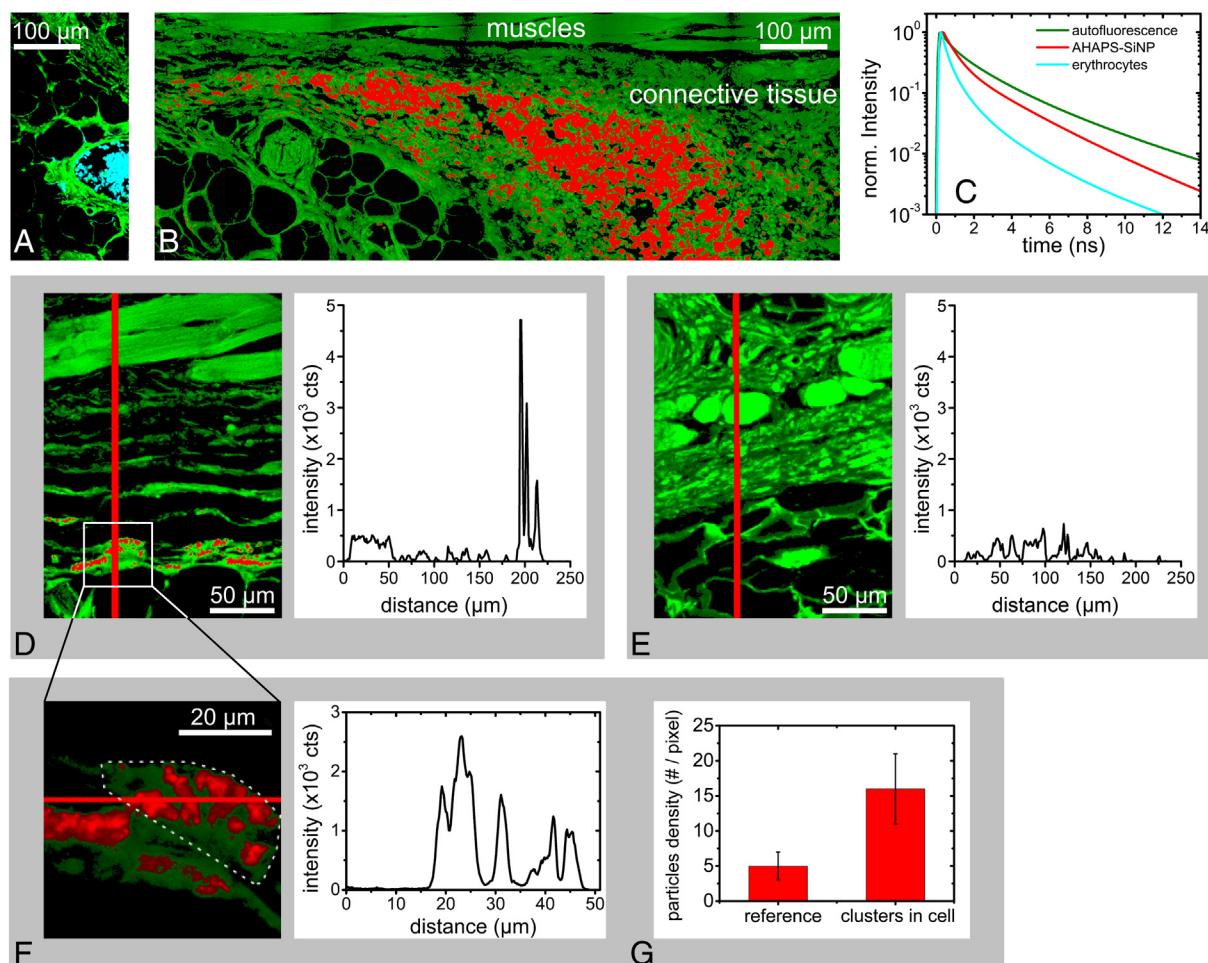


Figure 5. FLIM image analysis of mouse skin sections from group 8: **A-C** (top panel): **(A)** False-colored image of a section without AHAPS-SiO₂-NP (left flank). Tissue autofluorescence was color coded in green and erythrocyte autofluorescence in cyan. **(B)** False-colored image of a section from AHAPS-SiO₂-NP injection site. Tissue autofluorescence was color coded in green and fluorescence from AHAPS-SiO₂-NP in red. The high density of AHAPS-SiO₂-NP at the right half on the image probably represented the site of injection. **(C)** Fluorescence lifetime curves corresponding to the color coded areas in panels **A** and **B**. **(D and E)** Intensity line scans (red lines) of an image with **(D)** and without AHAPS-SiO₂-NP **(E)** with corresponding intensity profiles. Intensity in muscle was around 500 counts, in connective tissue below 500 counts and the AHAPS-SiO₂-NP had intensities well above 1000 and up to 5000 counts. **(F)** Measurement of area in panel **D** indicated by a white box (spatial resolution increased by factor 5). The image showed various distinct AHAPS-SiO₂-NP clusters presumably within macrophages. The contour of a selected macrophage is indicated by the dashed white line. The red line indicates a representative line scan, the corresponding histogram is shown in the right panel. Peak intensities for pixels containing AHAPS-SiO₂-NP ranged from 750 to 2300 counts. **(G)** Histogram of the average number of AHAPS-SiO₂-NP per pixel. For the reference sample embedded in an agarose gel (2.5 µg/µl AHAPS-SiO₂-NP) an average of 5 NP per pixel was calculated whereas 15 NP were found on average inside a pixel of the AHAPS-SiO₂-NP clusters within the macrophage highlighted in panel **F**. As the NP clusters inside this macrophage comprised a total of 3626 pixels, the number of AHAPS-SiO₂-NP taken up by this macrophage was estimated to be 56,400, corresponding to approximately 9.4 picograms of AHAPS-SiO₂-NP per macrophage.

which supported our decision to use this solvent. Clearly, both the vehicle and the structure, morphology and charge of NP have to be considered for understanding the permeation pathways.³³

We failed to see any cellular uptake of the AHAPS-SiO₂-NP by keratinocytes in vivo after topical application. The observed cellular uptake of SiO₂-NP by alleged Langerhans cells and not further specified skin cells in two previous in vivo studies^{14,15} may have also resulted from the different vehicles used as discussed above. Cellular uptake of SiO₂-NP has been shown in vitro for immortalized human and murine keratinocytes.^{45,46} Another study has addressed the discrepancy in the uptake capacity of immortalized dermal cell lines and primary keratinocytes. Only small amounts of positively charged SiO₂-

NP were taken up by primary keratinocytes compared to primary phagocytizing cells (Langerhans cells) and immortalized human keratinocytes that internalized larger numbers of SiO₂-NP depending on the size and surface charge.³⁶ Despite an enhanced cellular in vitro uptake of positively charged SiO₂-NP^{7,36} reduced cytotoxicity was observed in functionalized SiO₂-NP in comparison to the unfunctionalized NP.⁴⁵

Cytotoxic effects of SiO₂-NP in murine macrophages have been shown to correlate with surface properties of the NP in vitro.⁴⁷ In that study, amine- or carboxyl-functionalization of SiO₂-NP clearly reduced their cytotoxicity. This was explained by a lack of nuclear location of functionalized SiO₂-NP in contrast to unfunctionalized SiO₂-NP. This notion is supported

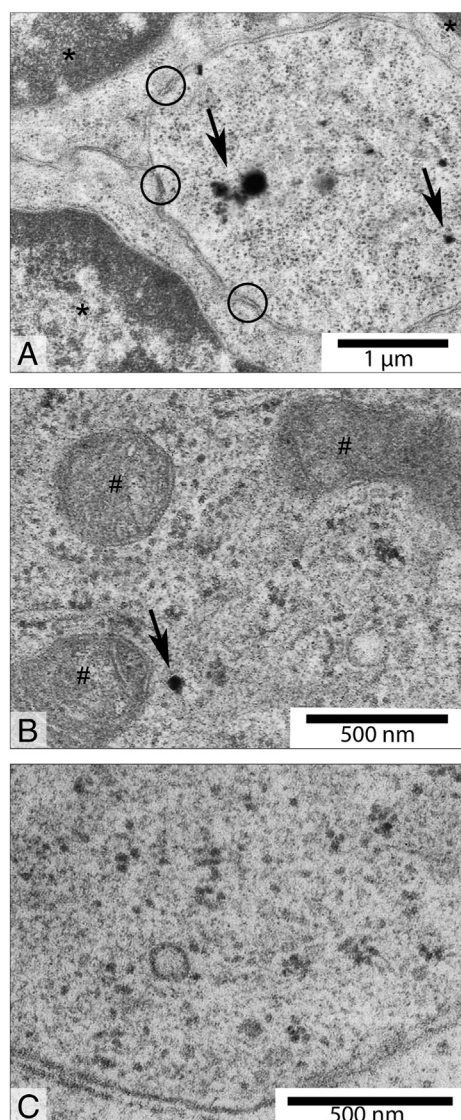


Figure 6. TEM analysis of a RLN localized the AHAPS-SiO₂-NP in cells without any subcellular adverse effects. Within the RLN the particles (arrows) were localized intracellularly either in clusters or as single particles. The uptaking cells were in close contact (open circle, structures consistent with immunological synapses) with lymphocytes (asterisks) and connect to these via immunological synapses, consistent with the uptaking cells being macrophages (A). Despite their intracellular localization SiO₂-NP obviously failed to induce any cellular changes, e.g., no ER or mitochondrial swelling was observed (hash key = mitochondria) (B). No SiO₂-NP or particle-like structures were detected in cells of the non-RLN (C).

were seen for silica-coated iron oxide NP by immunofluorescent labeling of macrophage marker CD169.⁴⁸ CD204 has previously been shown to be involved in the uptake of titanium dioxide, silica, diesel particles and latex beads in alveolar macrophages in a hamster model.⁴⁹

Toxicopathologic examinations by expert pathologists failed to reveal adverse effects in organs with AHAPS-SiO₂-NP as it has previously been reported for other SiO₂-NP.^{3,37}

Taken together, AHAPS-SiO₂-NP appear not to penetrate through the murine skin barrier even if the skin barrier is impaired. Of note, rodent skin usually shows higher permeation rates than the much thicker human skin and it thus appears even less likely that AHAPS-SiO₂-NP would penetrate through human skin.⁵⁰

Only after s.c. injection the SiO₂-NP were taken up by macrophages and during the course of 5 days translocated to the RLN. No distribution beyond the RLN was observed. Despite the cellular uptake of AHAPS-SiO₂-NP at the subcutaneous injection site as early as 24 h no particles were detected in the RLN at this time. The most likely explanation for this discrepancy is that phagocytosis and transmigration to the lymph node probably take more than this time.⁴⁴ It cannot be excluded, however, that a few first particle-carrying macrophages or dendritic cells may have already arrived at the RLN at this time, the number of which remaining under the detection level of the methods used here. Importantly, no toxic or other adverse effects were observed histologically in any of the tissues and time points investigated except for the infiltration of macrophages at the site of injection.

In summary, for biomedical concepts where targeting through the intact or inflamed skin is desired, the use of AHAPS-SiO₂-NP should be further investigated. However, it seems that for vaccination purposes where deeper entry to the epidermis, dermis or hair follicles is desirable, other routes of application, formulation or functionalization of SiO₂-NP may have to be established. Conversely, the lack of penetration of AHAPS-SiO₂-NP through the cornified skin layer may be exploited in applications where the advantages of AHPAS functionalization can be used, e.g., for the deposition of drugs or skin protective compounds that are not supposed to be absorbed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.nano.2014.04.004>.

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by our results for AHAPS-SiO₂-NP. Despite their intracellular localization no ultrastructural pathologic effects, e.g., mitochondrial or endoplasmic reticulum swelling were observed in cells of the RLN. Following subcutaneous injection the AHAPS-SiO₂-NP were taken up by macrophages as the immunofluorescent labeling of F4/80, CD204 and MHC class II indicated. FLIM images clearly identified the intracellular location within distinct compartments of the macrophage. Analysis of the FLIM images revealed an average concentration of approximately 60,000 AHAPS-SiO₂-NP per macrophage. Similar results in the RLN

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