In vitro translocation of quantum dots and influence of oxidative stress

Jorina Geys,1 Rita De Vos,2 Benoit Nemery,1 and Peter H. M. Hoet1

1Laboratory of Pneumology, Unit for Lung Toxicology, and 2Morphology and Molecular Pathology Section, Katholieke Universiteit Leuven, Leuven, Belgium

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Geys J, De Vos R, Nemery B, Hoet PH. In vitro translocation of quantum dots and influence of oxidative stress. Am J Physiol Lung Cell Mol Physiol 297: L903–L911, 2009. First published September 4, 2009; doi:10.1152/ajplung.00029.2009.—In vivo, translocation of inhaled nanoparticles to the circulation has been demonstrated. However, the interaction of nanoparticles with the lung epithelium is not understood. In this study, we investigated, in vitro, the translocation of nano-sized quantum dots (QDs; 25 pmol/ml) through a tight monolayer of primary isolated rat alveolar epithelial cells. The influence of surface charge on translocation was examined using nonfunctionalized QDs, amine-QDs, and carboxyl-QDs. The interaction between nanoparticles and the lung epithelium was monitored by repeatedly measuring the transepithelial electrical resistance (TEER) and by examining the cell layer with confocal microscopy. The effect of oxidative stress was tested by incubating the cells with tert-butyl hydroperoxide (t-BOOH; 75 μM or 1 or 10 mM); the antioxidant N-acetyl-L-cysteine was also used to assess the role of particle-mediated oxidative stress. No translocation through a tight monolayer of primary rat alveolar epithelial cells was observed for any of the different types of QDs. In general, an increase in TEER was found after incubation with QDs. A condition of low oxidative stress did not enhance translocation. In contrast, conditions of high stress (1 or 10 mM t-BOOH or due to QDs toxicity) with disruption of the cell layer, as shown in a decreased TEER, resulted in substantial translocation. In conclusion, no translocation of QDs was found through a tight monolayer of primary rat alveolar epithelial cells, regardless of the QDs surface charge. QDs did not impair the barrier function of the epithelial cells. In conditions with disruption of the cell-cell barrier, translocation was demonstrated.

nanoparticles; rat type II pneumocytes; surface charge; hydroperoxide; pulmonary epithelium

ENGINEERED NANOMATERIALS, i.e., materials with at least one dimension <100 nm (44a), are produced to exploit the properties and functions associated with their small size. Different types of engineered nanoparticles are already being used in electronics, tires, clothes, paints, cosmetics, and sunscreens. It is expected that engineered nanoparticles will be increasingly utilized in medicine, mainly for purposes of diagnosis, imaging, or drug delivery (17). However, engineered nanoparticles share the dimensions of ambient ultrafine particles, which are an important concern in environmental health. Ambient particulate matter has been associated with increased pulmonary and cardiovascular morbidity and mortality (11, 41, 43). Moreover, translocation of inhaled ultrafine particles to the systemic circulation has been shown in some (28, 35, 38, 40) but not all experiments (6, 32).

Further knowledge is necessary on the interaction of nanoparticles with the lung epithelium and the parameters that may play a role in their passage through the epithelium. Several studies have already indicated that physicochemical properties, such as size, shape, surface charge, and coating, are important factors determining particle toxicity (18, 29, 36, 37, 46). Furthermore, epidemiological studies have indicated that individuals with a preexisting pulmonary disease are more susceptible to the adverse health effects of inhaled particulates (5, 42, 47). Experimental studies strengthened this suggestion, showing that lung epithelial cells or macrophages “primed” with TNF-α or LPS, respectively, had an increased inflammatory response on exposure to particulate matter (22, 23, 48). In vivo, 14-nm carbon black particles aggravated lung inflammation related to bacterial endotoxin (24); moreover, pretreatment with LPS increased the extent of extrapulmonary translocation of particles into the blood (9).

In this study, we investigated the translocation phenomenon of nanoparticles, using quantum dots (QDs) in a recently developed in vitro model of the pulmonary epithelium, consisting of primary isolated rat alveolar epithelial cells seeded on a permeable membrane (13, 14). The study had four objectives: 1) quantification of the translocation of the QDs (15), 2) assessing the interaction between the nanoparticles and the lung epithelium, 3) investigation of the influence of particle surface charge on translocation, and 4) testing the influence of oxidative stress on translocation.

MATERIALS AND METHODS

All experimental protocols were performed in adherence with and approved by the Animal Care and Use Ethical Committee of the Katholieke Universiteit Leuven (K. U. Leuven) Medical Faculty (License P047-2004). The Laboratory of Pneumology, Unit for Lung Toxicology, is accredited by the Federal Authorities (License LA1210203). The Animal Facility has the obligatory accreditation of the authorized Federal Ministries (License LA2210393).

Experimental Setup

Primary alveolar epithelial cells were seeded on the permeable membrane of Transwell inserts, creating an apical compartment above the membrane and a basolateral compartment on the other side. Tight monolayers of primary alveolar epithelial cells were incubated with three types of QDs, each with different surface charge. Twenty-four hours after incubation, the amount of QDs translocation was quantified. The effect of nanoparticles on the cell monolayer was checked by repeatedly measuring the transepithelial electrical resistance (TEER) and by examination of the cell layer with confocal microscopy. Furthermore, the influence of particle concentration and oxidative stress on translocation was examined.

Nanoparticles

We purchased fluorescent Fort Orange [emission (em) 600 ± 10 nm] Type 2 EviTags from Evident Technologies in a solution of 12 nmol/ml QDs. To investigate the effect of surface modification, we used nonfunctionalized QDs (nF-QDs; Cd content is 0.11 μg/pmol...
QDs, amine-QDs (Cd content is 0.16 μg/pmol QD), and carboxyl-QDs (Cd content is 0.07 μg/pmol QD), all core/shell QDs consisting of CdSe/ZnS with a hydrodynamic diameter of ~25 nm. Before use, the QD stock solution was vortexed to reduce nonspecific aggregation; the QDs were diluted in HBSS with Ca²⁺ and Mg²⁺ (HBSS⁺; Invitrogen, Merelbeke, Belgium) containing 1% BSA (Sigma, Bornem, Belgium) to a final concentration of 25 pmol/ml QDs, and 200 μl of this suspension was added to the apical compartment of the Transwell.

**Particle characterization.** Particle size and distribution of the original solution and the concentrated solution were measured by dynamic light scattering using a Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, United Kingdom). To this end, 2 μl particles were dispersed in 800 μl of water, HBSS⁺, saline (0.9% NaCl), DMEM culture medium (Gibco), or HBSS⁻ containing 1% BSA. Three series of 15 measurements were performed, and the average particle size was calculated. HBSS⁺ containing 1% BSA was also run without particles. The zeta potential was calculated from electrophoretic mobility measurements in 5 series of 30 measurements.

**Stability of QD suspension.** The stability of the QDs was assessed by measuring the fluorescence of the QDs over time using a spectrophotofluorometer (SpectraMax Gemini; Molecular Devices). The nF-QDs, amine-QDs, or carboxyl-QDs were suspended in HBSS⁺ containing 1% BSA. In some instances, 75 μM or 10 mM tert-butyl hydroperoxide (t-BOOH; Sigma) was added. The fluorescence spectrum [excitation (ex) 405 nm / em 570 nm – 630 nm] was assessed immediately, 24 or 48 h after the solutions had been kept in the dark at room temperature, the corresponding medium serving as negative control.

**Isolation of Primary Alveolar Epithelial Cells**

The previous method of our laboratory (20) was used to isolate alveolar epithelial cells from the rat lung. Briefly, male Wistar rats of 190 g (Janvier) were anesthetized with an intraperitoneal injection of Nembutal (1.5 mg/kg pentobarbital; CEVA Santé Animale, Brussels, Belgium). The rats were exsanguinated and then tracheostomized. The lungs were perfused with 0.9% NaCl via the pulmonary artery. Finally, the heart was removed, and the lungs together with the trachea were cut off. The lungs were filled every 0.75 min with trypsin (250 mg in 100-ml HBSS⁺, pH 7.4; trypsin type I; Sigma). Thereafter, the lungs were filled every 5 min with trypsin and incubated at 37°C for 30 min. The trachea and bronchi were removed, and the lungs were cut for 5 min until fragments <1 mm³ were obtained. Fetal calf serum (5 ml; Invitrogen) and DNase I (1 mg; Roche Molecular Biochemicals, Mannheim, Germany) were added, and the suspension was shaken for 5 min. After filtering through cotton gauze and a 40-μm nylon filter (Falcon; Becton Dickinson, Leuven, Belgium), the cell suspension was centrifuged for 30 min at 250 g on a discontinuous Percoll gradient (p = 1.089 and 1.040 g/ml; Sigma). The band on the interface was collected and pelleted by centrifugation for 10 min at 250 g. The pellet of cells was resuspended in Waymouth medium (Invitrogen) with 10% fetal calf serum, 1% nonessential amino acids, and antibiotics. After 2 days, the cells were rinsed with HBSS⁺ and exposed to t-BOOH (10–500 μM; duplicate in 2 individual experiments) for 30 min or 24 h; after 24 h, the cell viability was measured using the MTT test. Briefly, after removing the medium and rinsing with HBSS⁺, the MTT solution (0.5 mg/ml MTT) was added for 3 h. Thereafter, the MTT solution was removed, and 100 μl of DMSO was added. The optical density was read at 550 nm with 655 nm as reference wavelength, using a microplate reader (Bio-Rad). Cells not incubated with t-BOOH served as 100% viability control (n = 4–6).

**Cytotoxicity of t-BOOH**

In preliminary experiments, the cytotoxicity of t-BOOH was assessed. Primary alveolar epithelial cells were seeded at a density of 0.75 × 10⁶ per square centimeter in Waymouth medium supplemented with 10% fetal calf serum, 1% nonessential amino acids, and antibiotics. After 2 days, the cells were rinsed with HBSS⁺ and incubated with t-BOOH (20 μl of 10 mM in 200 μl) or through pretreatment of the cells for 1 h with 10 mM t-BOOH (20 μl of 100 mM in 200 μl) in the apical compartment; conditions of (presumed) low oxidative stress was tested by incubating the cells with 75 μM (20 μl of 750 μM in 200 μl) t-BOOH in HBSS⁺ containing 1% BSA in the apical compartment; conditions of (presumed) high oxidative stress were tested by incubating the cells with 1 mM t-BOOH (20 μl of 10 mM in 200 μl) or through pretreatment of the cells for 1 h with 10 mM t-BOOH (20 μl of 100 mM in 200 μl) in the apical compartment. Additionally, cells were pretreated with 1 mM N-acetyl-l-cysteine (NAC; Sigma) for 1 h, followed by maintaining 1 mM NAC with or without 75 μM t-BOOH in HBSS⁺ containing 1% BSA (apical compartment). The effect of particle concentration on translocation was tested using 25, 40, 50, and 75 pmol/ml QDs, followed by a 3-[(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay (see Cytotoxicity of t-BOOH).

All culture conditions were performed in duplicate in three independent experiments. The effect of particle concentration and the incubation of QDs on the permeable membrane without a cell layer were done in duplicate in two separate experiments. Results are expressed as means ± SD.

**Measurement of TEER.** The integrity of the monolayer was checked by measuring the TEER, using the epithelial voltohmeter (EVOM) supplied with EndOhm chambers (World Precision Instruments). The resistance of a cell-free Transwell insert (19.7 ± 0.71 Ω·cm²) was subtracted from the resistance measured across each cell layer to yield the TEER value (Ω·cm²) of the cell monolayer. Before the incubation with particles and after collecting the particles, the TEER was measured 20 min after refreshing the culture medium. The influence of the particles on the integrity of the monolayer was examined through repeated measurements of the TEER, immediately after adding the particles (time 0) and at intervals of 0.5 or 1 h; changes in TEER are expressed relative to 30-min incubation of particles. All cell cultures had a TEER >200 Ω·cm² at the beginning of the experiment.
Sodium fluorescein leakage. Sodium fluorescein (1 mg/ml in HBSS +, 200 μl), a marker for paracellular transport, was added in the apical compartment, with 1,000-μl HBSS + being in the basolateral compartment. After placing the cultures for 60 min in the CO2 incubator (5% CO2, 37°C) samples were taken from the basolateral compartment, and the amount of sodium fluorescein was measured by spectrophotometry at 486 nm (Beckman DU-65 spectrophotometer). The concentration in the samples was calculated using a standard curve with known concentrations of sodium fluorescein (10, 2, 0.4, 0.08, and 0.004 μg/ml). The sodium fluorescein leakage was measured at the end of the translocation study.

Quantification of Translocation

In a first attempt to quantify the translocation of QDs after 24-h incubation, the amount of fluorescent QDs was measured using a spectrophotofluorometer (SpectraMax Gemini; Molecular Devices). A standard curve was obtained by suspending the nF-QDs, amine-QDs, or carboxyl-QDs in RPMI medium (without phenol red) containing 5% nonessential amino acids and 2% essential amino acids in a dilution range of 25 to 0.0061 pmol/ml (n = 2; 200 μl). The fluorescence was measured at 612 nm, with ex 485 nm; the corresponding medium served as negative control.

For elemental analysis, the medium of the apical and basolateral compartment was collected and dissolved in 3-ml ultrapure nitric acid (Sigma) in a waterbath at 80°C. The cadmium concentration was measured using inductively coupled plasma optical emission spectrometry. Determinations were made using ICP multi-element standard solution IV CertiPUR measured at the end of the translocation study.

Transmission Electron Microscopy

The morphology of the primary alveolar epithelial cells in culture was investigated with transmission electron microscopy. At the end of the study (3 days after seeding), the cells on the inserts were fixed with 2.5% glutaraldehyde-0.1 M phosphate buffer, pH 7.2, at 4°C. Post-fixation was carried out in 2% osmium tetroxide, 0.1 M phosphate buffer, pH 7.2. Dehydration was performed in graded ethanol series consisting of 30, 50, and 70% ethanol. Afterward, the inserts were incubated in a 2:1 mixture of ethanol and LR White (Electron Microscopy Sciences, Fort Washington, PA), rinsed twice in LR White, and further polymerized in LR White (39). Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined on a Zeiss EM 900 electron microscope. Images were recorded digitally with a Jenoptik ProgRes C14 camera system operated using Imaging-Pro Express software.

RESULTS

Particle Characterization

The size and zeta potential of the QDs were measured in different vehicles, as listed in Table 1. According to the technical information and the material safety data sheets provided by the producer, the QDs used have a hydrodynamic diameter of ~25 nm. The nF-QDs, amine-QDs, and carboxyl-QDs were found to differ in diameter depending on the vehicle and showed a tendency to agglomerate. The peak at 9.3 ± 0.3 nm originates from the BSA in the vehicle (vehicle alone, data not shown).

Stability of QD Suspension

The fluorescence peak emission was 606 nm for nF-QDs and 596 nm for carboxyl-QDs. There was a noticeable decrease in fluorescence intensity between the different vehicles, as listed in Table 1. According to the technical information and the material safety data sheets provided by the producer, the QDs used have a hydrodynamic diameter of ~25 nm. The nF-QDs, amine-QDs, and carboxyl-QDs were found to differ in diameter depending on the vehicle and showed a tendency to agglomerate. The peak at 9.3 ± 0.3 nm originates from the BSA in the vehicle (vehicle alone, data not shown). In water, the three types of QDs had a different zeta potential, with the nF-QDs being almost uncharged, the amine-QDs moderately negatively charged, and the carboxyl-QDs being more negatively charged. However, in HBSS+, + 1% BSA, this difference diminished, and all three types of QDs had a zeta potential around −10 mV.

Table 1. Physical characterization of the quantum dots

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>Diameter, nm</th>
<th>PDI</th>
<th>Zeta Potential, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>nF-QDs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>45.3 &amp; 174.6*</td>
<td>0.415</td>
<td>−2.7</td>
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<tr>
<td>Saline</td>
<td>121.4</td>
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<td>−1.9</td>
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<td>−5.1</td>
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<tr>
<td>DMEM</td>
<td>76.9</td>
<td>0.252</td>
<td>−1.7</td>
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<tr>
<td>HBSS + 1% BSA</td>
<td>9.0 &amp; 80.7**</td>
<td>0.473</td>
<td>−10.7</td>
</tr>
<tr>
<td>Amine-QDs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>20.4 &amp; 182.3*</td>
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<td>−32.6</td>
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<td>Saline</td>
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<td>−5.1</td>
</tr>
<tr>
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<td>−7.3</td>
</tr>
<tr>
<td>DMEM</td>
<td>33.0 &amp; 230.1*</td>
<td>0.490</td>
<td>−1.1</td>
</tr>
<tr>
<td>HBSS + 1% BSA</td>
<td>9.6 &amp; 648.2**</td>
<td>0.256</td>
<td>−8.3</td>
</tr>
<tr>
<td>Carboxyl-QDs</td>
<td></td>
<td></td>
<td></td>
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<td>0.253</td>
<td>−47.6</td>
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<tr>
<td>Saline</td>
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<td>0.245</td>
<td>−3.1</td>
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<tr>
<td>HBSS</td>
<td>114.2</td>
<td>0.407</td>
<td>−3.2</td>
</tr>
<tr>
<td>DMEM</td>
<td>33.2 &amp; 123.7*</td>
<td>0.356</td>
<td>−5.4</td>
</tr>
<tr>
<td>HBSS + 1% BSA</td>
<td>9.4 &amp; 129.8**</td>
<td>0.327</td>
<td>−12.4</td>
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</table>

Average diameter is from intensity distribution, measured by dynamic light scattering. PDI, polydispersity index; nF-QDs, nonfunctionalized quantum dots. *Samples are polydispersed with the coexistence of 2 or 3 particle sizes. **The intensity at 9.3 ± 0.3 nm originates from the BSA in the vehicle.
different types of QDs, with amine-QDs > (1.1-fold) nF-QDs > (5-fold) carboxyl-QDs. Although the peak emission wavelength was stable for up to 48 h, the fluorescence intensity of nF-QDs and carboxyl-QDs decreased by 20% after 24 h and then remained stable up to 48 h. The addition of 75 μM or 1 mM t-BOOH caused an immediate 10% decrease in fluorescence intensity compared with the control condition; after 24 and 48 h, 75 μM t-BOOH caused a 40% decrease, whereas addition of 1 mM t-BOOH resulted in a 70% decrease of peak emission fluorescence intensity. In all conditions, no shift in peak emission wavelength was noted.

**Cytotoxicity of t-BOOH or QDs**

Twenty-four hours after 30-min incubation with 500 μM t-BOOH, the relative viability of primary alveolar epithelial cells was decreased by 35.5% (Fig. 1A). Concentrations of 25–100 μM t-BOOH were not cytotoxic after 30-min or 24-h incubation.

Using the MTT viability assay, the amine-QDs and carboxyl-QDs were not cytotoxic after 24-h incubation, ≤75 pmol/ml QDs. However, for the nF-QDs, a dose-dependent cytotoxicity was obtained with relative viability <50% at 50 or 75 pmol/ml nF-QDs (Fig. 1B).

**Effect of QDs and Culture Conditions on Integrity of Cell Monolayer**

All monolayers of primary alveolar epithelial cells had a high TEER of 321 ± 90 Ω·cm² on day 2 after seeding, as measured 20 min after refreshing the culture medium. Manipulating the cells and changing the culture medium caused an immediate decrease to 231 ± 66 Ω·cm² for all conditions tested (with or without particles), but the TEER remained stable during the following 20–30 min (data not shown). Figure 2 shows the influence of QDs and culture conditions on the TEER, expressed relative to the TEER measured 30 min after adding the particles or the control medium. The TEER was not affected by 75 μM t-BOOH or incubation with NAC. Incubating the QDs on a cell-free membrane did not influence the resistance over the membrane (data not shown).

When primary alveolar epithelial cell monolayers were exposed for 24 h to 25 pmol/ml nF-QDs, amine-QDs, or carboxyl-QDs, obvious differences were noted between the separate experiments. In two of three experiments, the QDs caused an overnight increase in TEER (≤350%, between 6- and 21-h incubation), whereas in one experiment no such effect on TEER was noted. No significant differences could be demonstrated among the different culture conditions, although the mean relative TEER increased with incubation of QDs except in the presence of the antioxidant NAC (Fig. 2). Simulating a condition of high oxidative stress with 1 or 10 mM t-BOOH led to a decrease in TEER to almost 0 within 1 h (data not shown).

When different concentrations (25, 40, 50, or 75 pmol/ml) of QDs were incubated, the nF-QDs caused a decrease in TEER to almost 0 at 50 and 75 pmol/ml, whereas the amine-QDs and carboxyl-QDs did not (data not shown).

In addition to measuring the TEER, the leakage of sodium fluorescein was assessed in some experiments. In the control situation or condition of low oxidative stress (75 μM t-BOOH) or antioxidant (NAC, or NAC and 75 μM t-BOOH), leakage of sodium fluorescein <0.2% was found, both in the absence and presence of 25 pmol/ml QDs. In the case of high oxidative stress (1 or 10 mM t-BOOH), the leakage of sodium fluorescein was identical to that through a cell-free membrane (data not shown).

**Translocation of QDs and Effect of Oxidative Stress**

The detection limits in medium as applied in the basolateral compartment measured by spectrofluorometry were 6.25, 1.56, and 8.33 pmol/ml for nF-QDs, amine-QDs, and carboxyl-QDs, respectively. Using ICP-OES, lower detection limits of 0.117, 0.059, and 0.234 pmol/ml were recorded for nF-QDs, amine-QDs, and carboxyl-QDs, respectively. As a result, translocation down to 1% of the applied apical concentration could theoretically be detected for all types of QDs when using ICP-OES.

First, the passage of the QDs across the Transwell permeable membrane without a cell monolayer was evaluated. Twenty-four hours after apical application, an equal distribution between apical and basolateral compartment was found for all three types of QDs, corresponding to a recovery of >80% of the incubated amount (Fig. 3A).

Incubation of 25 pmol/ml nF-QDs, amine-QDs, or carboxyl-QDs on a monolayer of alveolar epithelial cells resulted in >90% retrieval of the QDs in the apical compartment after 24 h and no detectable QDs in the basolateral compartment (Fig. 3B). Treating the cells with 75 μM t-BOOH and/or NAC did not affect the lack
of translocation (Fig. 3B). However, in the presence of high oxidative stress, ~30% of translocation was found (using ICP-OES), irrespective of the type of QDs (Fig. 4).

**Microscopy**

The morphology of the primary alveolar epithelial cells was investigated with transmission electron microscopy (Fig. 5, A–C). The cell culture seeded from primary isolated alveolar epithelial cells showed a mixed type I-type II phenotype, mimicking the alveolar epithelium in vivo.

Visualization of the alveolar epithelial cells with fluorescence confocal microscopy confirmed the presence of tight junctions between adjacent cells (Fig. 5D). No difference in ZO-1 expression was noticed between the control culture and cultures with 75 μM t-BOOH (data not shown). Despite the fact that a droplet of QDs on a microscope slide could be

![Fig. 2. Relative transepithelial electrical resistance (TEER) of primary alveolar epithelial cells incubated with QDs in various culture conditions. Primary alveolar epithelial cells, seeded on Transwell inserts with 0.4-μm pore size (0.33-cm² surface area), are incubated without particles (open symbols, dotted line) or with 25 pmol/ml nF-QDs (●), amine-QDs (●), or carboxyl-QDs (●) in 4 culture conditions: normal (A), with 75 μM t-BOOH (B), with 1 mM N-acetyl-L-cysteine (NAC; C), or with 1 mM NAC and 75 μM t-BOOH (D). The TEER is expressed relative to 30-min incubation of particles (added at time 0). Data represent means ± SD; n = 6 from 3 independent experiments.](image_url)

![Fig. 3. Translocation of QDs. nF-QDs, amine-QDs, or carboxyl-QDs (25 pmol/ml) were introduced at the apical compartment on the Transwell permeable membrane (0.4-μm pore size, 0.33-cm² surface area) without the presence of cells (A) or in the presence of a monolayer of primary alveolar epithelial cells (B), in various culture conditions: normal, with 75 μM t-BOOH, with 1 mM NAC, or with 1 mM NAC and 75 μM t-BOOH. The amount of cadmium in the apical (●) and basolateral (●) compartment after 24-h incubation was measured by inductively coupled plasma optical emission spectroscopy (ICP-OES) and is given as percentage of the administered apical concentration. The concentration of cadmium in the basolateral compartment was below the detection limit in all conditions in B. Data represent means ± SD; n = 4 from 2 independent experiments (A) or n = 6 from 3 independent experiments (B).](image_url)
translocation of inhaled ultrafine technetium-99m (\(^{99m}\text{Tc}\))-carbon particles (10–100 nm) occurred to the blood in healthy human volunteers. Also, in hamsters, \(^{99m}\text{Tc}\)-albumin particles (<80 nm) rapidly passed into the circulation after intratracheal instillation (38). Oberdörster et al. (40) demonstrated translocation with high liver accumulation 1 day after inhalation of \(^{13}\text{C}\) nanoparticles in rats. However, the issue of translocation is still under debate since not all studies reported translocation (6, 32). At this moment, the mechanisms and kinetics of particle translocation are not yet known. So, in this study, we aimed to investigate the translocation of specific nanoparticles using our previously established in vitro model of the pulmonary epithelium (13, 14).

We used primary isolated rat alveolar epithelial cells (mainly isolated as type II pneumocytes) seeded on a permeable membrane, which showed, after 2 days of culture, a mixed type I-type II phenotype. After 2 days in culture, those alveolar epithelial cells developed tight junctions, as measured by a high resistance (TEER) over the cell layer and as evidenced by the expression of the ZO-1 protein, which plays a central role in tight junction formation and organization. ZO-1 is a cytoplasmic protein that links the transmembrane proteins, such as occludin, to other cytoplasmic components of the tight junctions and to the actin cytoskeleton (12, 33). As epidemiological studies indicated that individuals with a preexisting pulmonary disease are more susceptible to the adverse health effects of inhaled particulates (5, 42, 47), we aimed to investigate whether this has also an impact on the degree of translocation through the lung epithelium. A condition of oxidative stress was induced by culturing the alveolar epithelial cells in the presence of t-BOOH (21). A condition of low stress was chosen to not cause cell injury and thus not to disrupt the

**DISCUSSION**

Recent studies indicated that inhaled ultrafine particles can pass into the circulation. Nemmar et al. (35) concluded that visualized in a concentration-dependent manner (data not shown), QDs incubated on alveolar epithelial cells could not be visualized due to a high background signal.

**Fig. 4. Translocation of QDs through a monolayer of primary alveolar epithelial cells in the presence of high concentration oxidant.** nF-QDs, amine-QDs, or carboxyl-QDs (25 pmol/ml) were introduced in the apical compartment on the Transwell permeable membrane (0.4-\(\mu\)m pore size, 0.33-cm\(^2\) surface area) covered by a monolayer of primary alveolar epithelial cells cultured in the presence of 1 mM t-BOOH or after 1-h pretreatment with 10 mM t-BOOH. The amount of cadmium in the apical and basolateral compartment after 24-h incubation was measured by ICP-OES and is given as percentage of the administered apical concentration. Data represent means ± SD; \(n = 6\) from 3 individual experiments.

**Fig. 5. Microscopy of alveolar epithelial cells.** A–C: transmission electron microscopy of primary isolated alveolar epithelial cells in control conditions cultured on Transwell permeable membrane (0.4-\(\mu\)m pore size, 0.33-cm\(^2\) surface area) at 3 days after seeding. The cells show a mixed type I-type II pneumocyte phenotype with typical laminar bodies in some cells, and also microvilli and caveolae (black arrows) are present at the "lumen" site of the cells. Tight junctions between adjacent cells can be found (white arrow). The polyester membrane (white right brace) is relative tick. D: confocal microscopy of primary alveolar epithelial cells cultured in control conditions on Transwell permeable membrane (0.4-\(\mu\)m pore size, 0.33-cm\(^2\) surface area). Tight junctions at the cell-cell borders are visualized with zona occludens-1 (ZO-1) and Alexa Fluor 488 secondary antibody (green). Scale bar = 20 \(\mu\)m, scale bar in inset = 10 \(\mu\)m.
tightness of the cell monolayer. Preliminary experiments indicated that 75 μM t-BOOH was not cytotoxic (as assessed by MTT) after 24 h and that this treatment did not cause a decrease in TEER. In contrast, conditions of (presumed) high oxidative stress (1 or 10 mM t-BOOH) caused a disruption of the cell layer. Studies involving H₂O₂-induced barrier dysfunction have shown that relatively high doses are required to induce barrier function damage in airway epithelial cells, which are normally exposed to relatively high levels of oxygen and have strong antioxidant defense capacity (4, 8).

In addition, the capacity of Calu-3 cells or primary alveolar epithelial cells to metabolize H₂O₂ has been demonstrated (4, 26). Also, a condition with the antioxidant NAC was studied to assess the role of particle-mediated oxidative stress. Lovric et al. (30) demonstrated that the reactive oxygen species induced by CdTe QDs could be scavenged by NAC. Furthermore, the cytotoxicity of cysteamine-CdTe QDs could be prevented by the addition and maintenance of NAC in the culture medium (29).

We studied the translocation of three types of commercially available QDs: nF-QDs and QDs coated with amine or carboxyl groups. The concentration of QDs used is in line with those used in the literature, 1.25–40 nM (10, 46, 54). To minimize the possible interference of the vehicle with the particles, the QDs were suspended in HBSS⁺, a buffered salt solution. However, to maintain the tightness of the cell monolayer, it was essential to add 1% BSA in the apical compartment, RPMI medium was used in the basolateral compartment of the Transwell. To quantify the QDs with spectrophotometry, media without phenol red were used. However, spectrophotometry proved to be a far less sensitive detection method compared with ICP-OES. Noticeable is the difference in concentration of Cd in the different types of QDs: the amine-QDs contain the most Cd, followed by the nF-QDs and finally the carboxyl-QDs. This order corresponds with the order of fluorescence intensity, indicating that there might be a difference in the concentration of the stock solution. Both the nF-QDs and amine-QDs have a peak emission at 606 nm, whereas the carboxyl-QDs have a peak emission at 596 nm. All three types of QDs proved to have a stable fluorescence spectrum, although a decrease in fluorescence intensity was observed in conditions of high concentration of oxidant. This intensity decrease might be attributed to the formation of lattice defects on the QDs, thereby causing additional nonradiative recombination pathways, as this has been reported for photo-induced quenching states (25). However, we cannot completely exclude the contribution of a size reduction of the QDs. Besides an altered emission wavelength, a size reduction would also result in the decrease of photon counts due to the diminished absorption cross-section of QDs as their size becomes <5 nm (50).

Finding a negative zeta potential for the “positively laden” amine-modified QDs (Table 1) may appear counter-intuitive at first sight. However, the zeta potential represents the overall charge of the sliding plane, i.e., the cloud of ions that are close to the surface of the particle; it does not depend on the charge of the particle itself (27). Positively charged particles interact with negatively charged ions, even in purified water. The zeta potential depends, among factors, on the density (concentration) of the dissolved ions and their charge (charge density) and the charge and charge density of the particle itself. We are not the first to report such a “surprising” negative zeta potential for particles with positively laden moieties on their surface (2, 7, 31).

Without the presence of a cell layer, an even distribution of the QDs across the permeable membrane was noted 24 h after incubation, justifying the use of a polystyrene membrane with pores of 0.4-μm diameter. Through the tight alveolar epithelial cell monolayer, no translocation was detected for all three types of QDs at 25 pmol/ml. This lack of translocation is in contrast with our previous results using polystyrene nanoparticles, where ~6% translocated through a tight Calu-3 monolayer (13). Recently, the trafficking of polystyrene particles across the alveolar epithelium was confirmed (51). For QDs, so far, only penetration through the skin has been studied. Ryman-Rasmussen et al. (45) reported that topical application of commercial QDs with different sizes (QD565 and QD655), shapes, and surface coatings to porcine skin flow-through diffusion cells resulted in the penetration of the intact stratum corneum barrier with localization of QDs in the underlying epidermal and dermal layers as early as 8 h after topical application. However, skin penetration of nail-shaped QD621-PEG was minimal after 24 h and limited primarily to the outer stratum corneum layers (54). Recently, in an in vivo mouse model, low levels of penetration of QDs were seen with preferential collection in folds and defects in the stratum corneum and in hair follicles. Exposure to ultraviolet radiation increased penetration (34). Uptake in macrophages was proven for carboxy-QDs but not for amine-QDs or organic-QDs (10). Zhang and Monteiro-Riviere (53) showed that the uptake of carboxy-modified QDs, in keratinocytes, is rather quick (<1 h) and that this endocytosis process is probably dependent on lipid rafts and the G protein-coupled receptor (GPCR) pathway (53).

In vivo, the role of pulmonary inflammation in enhancing extrapulmonary translocation was demonstrated for 125I-radio-labeled 56.4-nm polystyrene particles but not for 202-nm particles (9). In addition, carbon black nanoparticles of 14 nm aggravated LPS-elicited lung inflammation and pulmonary edema in mice (24). However, in our experiments, no increased translocation of QDs was found in a condition with 75 μM t-BOOH. The presence of an antioxidant did not influence translocation either. In conditions of high oxidative stress with 1 or 10 mM t-BOOH, 30% translocation of the measured cadmium (core QDs) was observed, suggesting that translocation can occur when the epithelial barrier is disrupted. A cytotoxic concentration of 50 or 75 pmol/ml nF-QDs also resulted in translocation through the disrupted alveolar epithelial cell layer. Since marked hotspots of deposition exist in the lung (1), local disturbance cannot be excluded.

The incubation of the QDs on the alveolar epithelial cells did not impair the barrier function of the alveolar epithelium, as no decrease in TEER was measured. In fact, the opposite was the case: a general increase in TEER might (purely speculative) reflect the cellular response to subtoxic stress. Our findings seem to be in contrast with those of Yacobi et al. (52), who demonstrated an acute and sustained decrease in TEER after exposure of rat alveolar epithelial cells to 176 μg/ml chitosan- or alginate-coated QDs, although it was reported that
lower concentrations did not decrease TEER significantly over 24 h of exposure. A dose-dependent decrease in TEER was also noted for Calu-3 cells exposed to multi-walled carbon nanotubes (44). The lack of sodium fluorescein flux in our experiments, a marker for paracellular transport, confirmed that the alveolar epithelial cells were not leaky.

Uptake of the QDs in the alveolar epithelial cells could not be visualized by confocal microscopy. However, it should be stressed that the visualization of single QDs requires a high brightness, which resulted in a huge background noise when working with fixed cells, so no adequate information could be obtained. Intracellular localization of QDs has been shown for human epidermal keratinocytes in which agglomerated QDs of different sizes were found in the cytoplasm, free or in membrane-bound vacuolar compartments (46, 54). In addition, in N9 cells, red cysteamine-CdTe QDs (5.2 nm) were found distributed throughout the cytoplasm, whereas green positively charged QDs (2.2 nm) were localized predominantly in the nuclear compartment within 1 h (29). No experimental explanation was given, however, it was suggested that differences in aggregation could account for the distinct subcellular localization.

This pulmonary permeability model can be used to study the mechanisms involved in the phenomenon of extrapulmonary translocation of nano-sized particles and to screen particles for translocation. We observed a marked difference in translocation between our previously investigated nano-sized polystyrene particles (13), which showed 6% translocation across a layer of Calu-3 cells, and the presently studied QDs, which, in a model using primary rat type II pneumocytes, did not translocate unless the cells were damaged. It remains to be seen whether the observed differences are due to the cell type studied or to the nature of the nanoparticles. The advantage of models is that they allow a simplification, but oversimplification is also their drawback. In the lung, there is interplay between different cells. In our experiments, we focused on the alveolar epithelium and a physiological condition of oxidative stress. Further refinements of the model can include the use of cocultures of epithelial and endothelial cells and/or macrophages (3, 16, 19).

In conclusion, low concentrations of different types of QDs did not impair the barrier function, nor did QDs translocate through a monolayer of primary rat alveolar epithelial cells, indicative for the absence of transcellular transport, in the given circumstances. However, in conditions with disruption of the epithelial barrier, translocation was demonstrated, indicative for paracellular transport.

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REFERENCES

IN VITRO TRANSLOCATION OF QUANTUM DOTS


