Translocation of SiO2-NPs across in vitro human bronchial epithelial monolayer

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Abstract. Safe development and application of nanotechnologies in many fields require better knowledge about their potential adverse effects on human health. Evidence of abilities of nanoparticles (NPs) to cross epithelial barriers and reach secondary organs via the bloodstream led us to investigate the translocation of SiO2 NPs of 50 nm (50 nm-SiO2-NPs) across human bronchial epithelial cells that are primary targets after exposure to inhaled NPs. We quantified the translocation of fluorescently labelled SiO2 NPs at non-cytotoxic concentrations (5 and 10 µg/cm²) across Calu-3 epithelial monolayer. After 14 days in culture Calu-3 cells seeded onto 3 µm-polycarbonate Transwell membranes formed an efficient bronchial barrier assessed by measurement of the transepithelial electric resistance and quantification of the permeability of the monolayer. After 24 hours of exposure, we observed a significant translocation of NPs that was more important when the initial NP concentration decreased. Confocal microscopy observations revealed NP uptake by cells and an important NP retention inside the porous membrane. In conclusion, 50 nm-SiO2-NPs can cross the human bronchial epithelial barrier without affecting the integrity of the epithelial cell monolayer.

1. Introduction

The production of nanomaterials and their use in many fields has considerably increased in the last decade. Nanomaterials are used in a large variety of applications such as electronics, cosmetics, drug delivery etc. Their widespread use presents a potential risk for human health due to unintentional environmental and/or occupational exposure [5]. The respiratory tract is one of the primary targets of NP exposure. It represents the major route for unwanted exposure with an important surface area of gas exchange that promotes NP penetration. According to their size, inhaled NPs could be deposited in three regions of the respiratory tract: nasopharyngeal, tracheobronchial and alveolar regions [10]. After inhalation, NPs can be eliminated by two types of clearance: i) mucociliary clearance for NPs trapped within the mucus in the airways and ii) alveolar-macrophage-clearance in the alveoli. However the uptake efficiency by alveolar macrophages is suspected to be lower for NPs than for larger particles [8]. Despite these defence systems, the smallest particles can persist throughout the respiratory system [5].

A key question in nanotoxicology concerns the NP abilities to cross epithelial barriers at the site of exposure [11]. Several biodistribution studies have shown that inhaled NPs can translocate...
through the respiratory barrier reaching the blood circulation and secondary organs. Although the amount of translocation is generally low (1% or less), chronic exposures could lead to an accumulation of biopersistent NPs in secondary organs [12, 13]. Translocation depends on NP size, composition, or surface charge [9]. For instance, a study of a panel of near infrared fluorescent NPs instilled in rats, have shown that NPs with a hydrodynamic size less than 34 nm rapidly moved from the lung to lymph nodes and that for particles below 34 nm, the presence of negative charges prevent their translocation [4].

With the increasing synthesis of a huge number of NPs differing in size, chemical composition or surface properties, a thorough assessment of their abilities to cross the different biological barriers has to be performed as well as the determination of the physicochemical characteristics favouring their translocation.

In this context, our aim was to develop an in vitro model of a human bronchial epithelial monolayer to study NP translocation. For this purpose we used the Calu-3 cell line grown in Transwell membranes and characterized for its ability to form an efficient barrier. Calu-3 monolayers were exposed for 24 hours to increasing concentrations of 50 nm-fluorescently labelled SiO$_2$ NPs used as a model of NPs. SiO$_2$ NP uptake by epithelial cells was investigated by confocal microscopy and their translocation through the epithelial monolayer was quantified measuring the fluorescence in the apical and basolateral compartments.

2. Materials and Methods

2.1. Nanoparticles

The fluorescein-doped 50 nm-SiO$_2$-NPs were synthesized as already described by Vranic et al., 2012 [14]. Briefly, fluorescein isothiocyanate (FITC) was covalently attached to (3-aminopropyl)-trimethoxysilane (APS) by reaction of the amino group with the isothiocyanate group. 5 mg of FITC was dissolved in 5 mL of 42.7 mM of APS in ethanol. After 12 hours of stirring, the fluorescent silane was added to 250 mL ethanol, 5 mL TEOS (Tetraethyl orthosilicate), 7.6 mL ammonium hydroxide (28 %) and 10.9 mL water. The reaction was performed at 50°C for 12 hours in the dark under magnetic stirring. The so-prepared particles have an average diameter of about 30 nm. Further silica growth was performed to obtain the expected diameter of 50 nm and to make the NP more spherical with a pure silica surface. Dynamic light scattering and zeta potential values were measured by Zetasizer (nano ZS, Malvern Instruments, USA) providing hydrodynamic diameter distribution curves showing small agglomerates (242.7 nm) and a negative surface charge in culture medium (zeta-potential of -9.8 mV).
2.2. Cell culture

2.2.1. Calu-3 cells
Human lung adenocarcinoma (Calu-3) cells were purchased from the American Type Culture Collection (ATCC, Sigma-Aldrich, Saint Quentin Fallavier, France) and grown in DMEM/F12 (Dulbecco’s Modified Eagle Medium) medium with phenol red (Life Technologies, Saint Aubin, France), containing 10% fetal calf serum (FCS, Life Technologies) and 1% glutaMAX (Life Technologies), subsequently referred to as complete cell culture medium. All experiments were performed with cells from passages 21 to 33. Cells were grown in T75-flasks (Costar, Sigma-Aldrich) for cellular expansion and onto 3 µm-polycarbonate Transwell membranes (12 mm in diameter) in two compartment chambers for translocation experiments. Cells were seeded at 500,000 cells/cm² on 3 µm polycarbonate Transwell membranes inside 12-wells plate (Costar, Sigma-Aldrich). 500 µL of complete culture medium were added in the apical chamber and 1,500 µL in the basolateral chamber. Cell cultures were maintained in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

2.2.2. Assessment of cell confluence
To assess Calu-3 confluence, the TransEpithelial Electrical Resistance (TEER) was measured and the monolayer permeability to Lucifer Yellow (LY) (Sigma-Aldrich, Saint Quentin, France) was assessed three times per week, during 14 days.
Before TEER measurements, cells were rinsed by fresh complete cell culture medium. After 1 hour of incubation at 37°C in fresh culture medium, the TEER values were determined using the electronic circuit of the Epithelial VoltOhmMeter (EVOM) and the STX2 electrode (World Precision Instruments, Hertfordshire, United Kingdom). Mean TEER value of ten wells was calculated. The mean resistance of a cell-free Transwell insert was subtracted from the resistance measured across each cellular monolayer to yield the TEER value of the cell monolayer.

For LY measurements, cells were rinsed by HBSS (with calcium and with magnesium) and incubated for 1 hour at 37°C with 500 µL of LY (0.2 µg/mL final in HBSS). FluoSart Galaxy (BMG, Ortenberg, Germany) was used to determine fluorescence (485 nm / 520 nm) of apical and basolateral media.

2.2.3. Viability assay
Viability of cell cultures was determined by the mitochondrial metabolic assay, WST-1 according to the method described by Hussain et al., 2010 [7].

2.3. Translocation studies
14-days confluent cultures were rinsed with HBSS (Sigma-Aldrich) to eliminate trace amounts of FCS. 50 nm-SiO₂-NP stock (23.2 mg/mL in water) was vortexed shortly before making final dilutions (5 or 10 µg/cm² corresponding to 10 or 20 µg/mL) in complete cell culture medium without phenol red and FCS.
After 24 hours of exposure, 100 µL of apical and basolateral media were deposited inside a white 96-well plate with clear bottom (Greiner, Courtaboeuf, France) as well as serial dilutions of 50 nm-SiO₂-
NPs (in a concentration range of 0 to 10 µg/cm²). Fluorescence was quantified with Infinite 200 Pro (TECAN, Lyon, France). 50 nm-SiO$_2$-NPs were excited at 488 nm and detected at 521 nm. Blank fluorescence value (medium without NPs) was removed from each fluorescence value obtained. Fluorescence values were converted in concentration values or in percentage values through the use of a standard curve. Results for the “filter” or “cells/filter” chambers were determined by subtracting values of apical and basal media from the initial NP concentration applied. Membranes were removed and mounted for imaging. For confocal microscopy experiments without cells, the membrane was observed through a reflection mode.

2.4. Immunofluorescence and Microscopy

After 24 hours of exposure, cells were fixed with paraformaldehyde 4% in PBS (Santa-Cruz, Heidelberg, Germany) for 20 minutes at room temperature and incubated for 10 minutes with NH$_4$Cl (50 mM, Sigma-Aldrich), permeabilized in 0.05% PBS-Tween 20 (Sigma-Aldrich) for 5 minutes. Fixed cells were incubated over night at 4°C with either MUC5AC or ZO-1 primary antibodies (Life Technologies) diluted to 1:500 followed by secondary antibodies stained by Alexa 488 or 568 fluorochromes (1:500, Life Technologies) for 2 hours at room temperature. For actin staining, fixed cells were incubated for 30 minutes with TRITC-phalloidin (0.9 nM in PBS, Life Technologies). Cell nuclei were stained with DAPI (4’,6-Diamidino-2-Phenylindole, Dihydrochloride, 0.25 µg/mL in PBS, Life Technologies) for 1 minute. After mounting in polyvinyl alcohol mounting medium with DABCO (Sigma-Aldrich), cells were examined under a Zeiss LSM 710 confocal microscope using objectives 40X and 63X. Image treatment was done with Image J software (Image J 1.42 NIH, USA).

2.5. Transmission Electron Microscopy

Cell cultures were fixed and analyzed by transmission electron microscopy according to the method described by Hussain et al., 2010 [7].

2.6. Statistical analysis

Every experiment was repeated at least twice with triplicates of each condition. Data are represented as means ± SD and were analyzed on commercially available software SigmaStat (Version 3.0, Systat software Inc, San Jose, California, USA) analysis of variance (one-way ANOVA) followed by Dunnett’s test for multiple comparisons with p<0.05 (two tailed) considered as significant.

3. Results

3.1 50 nm-SiO$_2$-NP distribution across 3 µm-polycarbonate Transwell membranes in absence of cells

Translocation could be studied using an epithelial monolayer seeded onto a porous membrane in an insert delimiting two chambers allowing to get access to the lower (basolateral) chamber where NPs
translocate after their application in the upper (apical) chamber. Membranes of different porosities are commercially available but the choice of the porosity is dictated by their abilities to allow NP translocation and cell development. Low porosity (0.4 µm) is better for cellular growth but proved to prevent an efficient Transwell crossing of the 50 nm-SiO$_2$-NPs (data not shown). We evaluate the suitability of 3 µm-polycarbonate Transwell membranes by quantifying NP concentrations in the apical and the basolateral chambers and by studying the NP behaviour inside the porous membrane by confocal microscopy after 24 hours of incubation.

As shown in Figure 1, 50 nm-SiO$_2$-NPs can cross the 3 µm-polycarbonate Transwell membranes after 24 hours of treatment. The NP concentration in the basolateral chamber increased as the NP concentration initially applied in the apical chamber increased (Fig. 1A). By contrast when results were expressed as percentage of translocation, we noted that the higher initial apical concentration led to a lower percentage of translocation. For concentrations of 5 or 10 µg/cm$^2$, we determined a translocation of 4.04% and 3.33% respectively (Fig. 1B) and an important trapping inside the filter of 91.08% and 92.47% respectively (Fig. 1B).

**Figure 1**: 50 nm-SiO$_2$-NP translocation study across 3 µm-polycarbonate Transwell membranes without cells, after 24 hours of incubation. (A) 50 nm-SiO$_2$-NP concentrations found in each chamber and in the porous membrane (filter) (in µg/cm$^2$); (B) 50 nm-SiO$_2$-NP distribution in each chamber (in percentage). $n=3$ for each condition; # indicates that the condition 10 µg/cm$^2$ is statistically different from the condition 5 µg/cm$^2$ ($p<0.05$)

NP retention inside 3 µm-polycarbonate Transwell membranes after 24 hours of incubation was confirmed by confocal microscopy observations. Figure 2 shows a large amount of NPs on the top of the membrane and to a lower amount inside and at the bottom of the membrane.

**Figure 2**: 50 nm-SiO$_2$-NPs retention on the top and on the bottom of a 3 µm-polycarbonate Transwell membrane after an incubation of 24 hours at the concentration of 10 µg/cm$^2$. SiO$_2$-NPs labelled in green and filter in red. Scale bar: 10 µm; X40.
3.2 Calu-3 epithelial monolayer

In order to evaluate the capacity of Calu-3 cells seeded onto 3 µm-polycarbonate Transwell membranes to form an efficient barrier, a time course study was performed over 14 days after seeding measuring the TEER and the epithelial permeability (Fig. 3A). The 3rd day TEER-value was around 100 Ω/cm² and progressively increased to reach 1,200 Ω/cm² at the 14th day. The passage of LY in the basolateral chamber progressively decreased from 800 AU at the 3rd day to 0 at the 14th day demonstrating an absence of paracellular transport across the cellular monolayer (Fig. 3A). The TEER increase associated to the decrease of LY flux over time shows the establishment of an efficient Calu-3 barrier. These functional studies were confirmed by ultrastructural observations of Calu-3 cells which exhibited cellular junctions between cells (Fig. 3B).

![Figure 3](image)

**Figure 3:** (A) TEER and Lucifer Yellow measurements. Calu-3 cells grown onto 3 µm-polycarbonate Transwell membranes for 3 to 14 days. (B) Observation by transmission electron microscopy of the ultrastructure of Calu-3 cells seeded onto 3 µm-polycarbonate Transwell membranes after 14 days of culture. The circles show different types of junctions.

Particularly, tight junctions are present as shown by confocal microscopy observations of the specific immunostaining with ZO-1, a polypeptide exclusively associated with tight junctions (Fig. 4A), in a confluent cellular monolayer shown by actin filament staining (Fig. 4B). Moreover, the culture of Calu-3 onto 3 µm-polycarbonate Transwell membranes allowed their differentiation as they express the mucin MUC5AC (Fig. 4C). For further studies, Calu-3 cells were only used after 14 days of culture.

![Figure 4](image)

**Figure 4:** Confocal microscopy observation of Calu-3 cells seeded onto 3 µm-polycarbonate Transwell membranes after 14 days of culture. (A) Tight junctions labelled in green by ZO-1 specific antibody (B) Actin filaments in red with TRITC-phalloidin and (C) Mucus in green by MUC5AC specific antibody. Cell nuclei labelled in blue with DAPI. Scale bar: 10 µm
3.3 50 nm-SiO₂-FITC-NP distribution across Calu-3 epithelium

A viability assay was performed showing that NPs were not cytotoxic for Calu-3 cells (data not shown). Furthermore observations by confocal microscopy revealed that the integrity of the monolayer was conserved although NPs were present inside the cells. This is confirmed by the staining of actin filaments which preserve a proper structure (Fig. 5B & 5D). Moreover NP uptake seemed to increase with the exposure-concentration (Fig. 5A & 5C). Orthogonal views of Z-stack merge showed the presence of NPs around cell nucleus and close to the basolateral level of the cell (Fig. 5B & 5D).

![Image of NP distribution across Calu-3 epithelium](image)

**Figure 5:** 50 nm-SiO₂-NP detection inside Calu-3 cells seeded onto 3 µm-polycarbonate Transwell membranes after an exposition of 24 hours at 5 µg/cm² (A: z-stack merge of green channel & B: z-stack merge of blue, red and green channels + Orthogonal Views) and 10 µg/cm² (C: z-stack merge of green channel & D: z-stack merge of blue, red and green channels + Orthogonal Views). NPs labelled in green, cell nuclei in blue, and actin filaments in red. Scale bar: 10 µm; X63.

After studying NP internalization by Calu-3 cells, NP translocation studies were carried out. A NP crossing through the Calu-3 monolayer model was observed. Basolateral NP concentrations were similar whatever the initial NP concentration was, around 0.2 µg/cm² (or 0.4 µg/mL) (Fig. 6A), and
NPs were mainly trapped within the “cells/filter” compartment (Fig. 6B). The percentage of NP translocation was 1.8 times more important when the cells were treated at 5 µg/cm² compared to a treatment at 10 µg/cm² (Fig. 6B).

**Figure 6:** 50 nm-SiO₂-NP translocation study across 3 µm-polycarbonate Transwell membranes with confluent cultures after 24 hours of treatment. (A) 50 nm-SiO₂-NP concentrations found in each compartment (in µg/cm²); (B) 50 nm-SiO₂-NP distribution in each compartment (in percentage). n=3 for each condition; # indicates that the condition 10 µg/cm² is statistically different from the condition 5 µg/cm² (p<0.05)

4. Discussion

In this study we provided evidence that Calu-3 cell line grown on 3 µm-polycarbonate Transwell membranes is a valuable model to investigate the NP translocation through the bronchial epithelial monolayer. We have shown that 50 nm-SiO₂-NPs were internalized and translocated to a low but significant amount after 24 hours of exposure.

To easily quantify NP translocation without time-consuming and cost-efficient techniques, we have chosen to use fluorescently labelled NPs. By comparison with other quantitative techniques such as ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometry) based on elemental analysis, the use of fluorescently labelled NPs allows a combination of experiments such as measurements of NP flux and morphological studies of NP internalization. 50 nm-SiO₂-NPs were chosen as a relevant NP model as they can be easily labelled with fluorochromes. Moreover they can be considered as relevant for human exposure as they are widely used in different industrial applications as well as in nanomedicine [2].

To characterize the NP abilities to cross physiological barriers after different routes of exposure, *in vitro* models have to be developed. To mimic the lung barrier, different cell lines were available such as Calu-3, NCI-H292, 16HBE14o- or A549. We have chosen the Calu-3 cell line as the other cell lines did not exhibit effective tight junctions when grown on 3 µm Transwell membranes [6] (Isabelle George personal communication). By contrast Calu-3 cells have the ability to form an efficient barrier developing tight junctions as we demonstrated by morphological (transmission electron microscopy and ZO-1 immunolabelling) and functional studies (TEER and LY flux). Moreover the Calu-3 cell line is known to express xenobiotic metabolizing enzymes such as CYP1A1 or CYP2B.
and to produce mucus (and more specifically the MUC5AC mucin) [1] as we confirmed in our cultures.

The use of 3 µm-polycarbonate Transwell membranes was a prerequisite as 0.4 µm membranes prevented 50 nm-SiO$_2$-NPs Transwell crossing (data not shown). A lower NP translocation through 0.4 µm-polycarbonate Transwell membranes was also observed in studies using Fluoresbrite® polystyrene latex NPs [3] or amine-modified or carboxyl-modified polystyrene beads [6]. Such membrane porosity seemed to be a good compromise as it allows combining a proper development of the Calu-3 cells with a detectable NP translocation. Nevertheless a large amount of NPs (around 91%) was retained inside the membrane, leading to an underestimation of the real NP flux to the basolateral chamber. The amount of NP translocation through the 3 µm-polycarbonate Transwell membranes was also shown to be low for 37 nm Fluoresbrite® polystyrene latex NPs [3] but to be more important for 46 nm amine-modified or carboxyl-modified polystyrene beads suspended in the presence of 1% bovine serum albumin (BSA) [6]. These data suggest that the NP composition, their size as well as conditions of exposure influence NP translocation. Moreover, we noticed that increasing NP concentrations reduced NP translocation, suggesting that at high concentrations NP agglomeration could be favoured preventing the efficient crossing of the membrane by NPs. This also shows that an increase of NP flux in the basolateral compartment cannot be achieved by increasing initial NP concentrations. Modifying the suspension medium adding BSA as Geys et al., 2006 or serum could increase NP translocation [6].

Using not cytotoxic concentrations, confocal microscopy experiments have revealed that 50 nm-SiO$_2$-NPs are internalized by the Calu-3 monolayer without inducing structural modifications of actin filaments after 24 hours of exposure. This is in agreement with another study using the same 50 nm-SiO$_2$-NPs that were shown to be dose-dependently internalized by NCIH-292 cells grown as islets on plastic (Sandra Vranic personal communication).

Our results demonstrated that exposing Calu-3 monolayers to relevant NP concentrations induced a low but a significant NP translocation (0.2 µg/cm$^2$ corresponding to 0.4 µg/mL). Considering the important NP retention inside the porous membrane that we noticed with experiments of NP flux in the absence of cells, the real translocation would likely be higher. However the respective NP distribution inside cells and membranes is unknown as the amount of NPs trapped in cells remains to be measured. The 1.9 to 3.5% of translocation that we observed through Calu-3 monolayer and membrane with 50 nm-SiO$_2$-NPs is in the same range (6%) as the one observed with the same cell line for 46 nm amine-modified or carboxyl-modified polystyrene beads [6].

To conclude, we have shown that the Calu-3 cell line proved to be a relevant in vitro model for the study of NP translocation across an in vitro human bronchial epithelial monolayer. Experiments are in progress to investigate the role of NP physicochemical characteristics and NP environment in NP translocation.
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